Characterisation of the Murine Gammaherpesvirus-68 M4 Gene

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PhD

University of Edinburgh

2002

Declaration

I declare that this thesis has been composed by myself and has not been submitted for any other degree. The work described herein is my own except where otherwise indicated and all work of other authors is duly acknowledged.

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Acknowledgements

I would like to thank Dr. Bernadette Dutia and Professor Tony Nash for their excellent supervision. I am indebted to Bernadette for all her time, patience and guidance, and Tony for his words of wisdom and for the opportunity to pursue my project.

I would like to acknowledge the Medical Research Council for funding my research.

Thanks to all the PhD students and the MHV-68 group. A special thank you to Debs, Heather, Alastair, Kev, and Bob for their advice, support and friendship. Furthermore, I would like to thank the entire department for their help.

A huge thanks to group "D" from the Graduate Schools Programme at Durham. The fun and mad games we shared along with their advice and understanding boosted my sanity and confidence. Thanks to them, I entered my final year with a positive and enthusiastic attitude.

Words cannot express the thanks I have for Adele, Jamie, Leyla, Dan, Charlotte, Suganya, and Matt for their invaluable encouragement and unconditional patience at various stages during my PhD. Cheers for all the beer, coffee, chocolate and hugs.

I could not have completed my PhD without Sarah and her amazing support, high spirits and belief in me, so a massive thanks to her.

Madeleine, Fiona, and Isobel injected much fun, laughter, and dancing into the many lonely days. So a very, very special thanks to them. I will never forget the hugs, friendship and experience we shared.

Thanks to Ali for his love and understanding during the run up to the viva and thanks to Cape Town for the refreshing break that I deserved.

Last of all, thanks to Dad, Sandra, and the family for having faith in me.

Abstract

Murine gammaherpesvirus 68 (MHV-68) is a lymphotropic virus which infects wild murid rodents and can readily infect experimental mice. Thus, MHV-68 is an invaluable small animal model for elucidating gammaherpesvirus pathogenesis and immune evasion strategies. The MHV-68 genome has been fully sequenced (Virgin *et al.*, 1997) and comparative sequence analysis has shown that it is more closely related to Kaposi's sarcoma-associated herpesvirus (KSHV) and herpesvirus saimiri (HVS) than Epstein-Barr virus (EBV). There are at least 80 open reading frames (ORFs) within the genome, many of which are present in other gammaherpesviruses. There are, however, 4 ORFs, termed M1-M4, which are unique to MHV-68. Gammaherpesviruses have the propensity to abduct immunomodulatory elements, therefore it is likely that such elements will be encoded within the MHV-68 genome. One such candidate is the M4 gene. The aim of the project was to functionally characterise the M4 gene and elucidate its role in the pathogenesis of MHV-68.

Half of the M4 protein was expressed in a bacterial expression system and the purified protein was used to generate anti-sera. Immunoprecipitation studies demonstrated the M4 sera bound to a protein of ~44kDa, the predicted size of the M4 product. Analysis of M4 transcription *in vitro* showed that the gene was transcribed both early, prior to DNA synthesis, and late in the virus lifecycle.

The M4 gene was cloned into a mammalian expression construct and transfected into COS-7 cells. Cells containing the construct were selected under G418 (neomycin derivative) antibiotic selection. However, we were unable to demonstrate that the COS-7 cells expressed the M4 protein.

The M4 gene was inserted into MHV-76, a virus which lacks part of the left hand end of the genome including the M4 gene, generating a M4 knock-in (M4KI) recombinant virus. Apart from this deletion, MHV-76 harbours the full contigency of MHV-68

genes. Polymerase chain reaction (PCR) and Southern analysis were used to demonstrate the presence of the M4 gene in the M4KI virus. Reverse trancription-PCR (RT-PCR) and northern analysis were used to show that the M4KI virus was expressing M4 RNA *in vitro*. The M4KI virus was used for comparative studies with MHV-76, which lacks the M4 gene, and MHV-68, which contains the M4 gene. The growth kinetics of MHV-76 are similar to MHV-68 *in vitro* but in contrast the virus appears to be cleared more efficiently *in vivo* in the lungs of mice. The establishment of latency is also impaired in the spleens of MHV-76-infected mice (Macrae *et al.*, 2001). *In vitro* studies revealed the M4KI virus growth kinetics were similar to MHV-68 and MHV-76. Infection of BALB/c mice with the M4KI virus revealed that it replicates in the lung with the same kinetics as MHV-76. Thus, compared to MHV-68, MHV-76 and M4KI virus titres rise slightly faster, and levels of virus are reduced or cleared quicker. M4KI virus infective centres were much reduced in the mediastinal lymph nodes in comparison to MHV-76 and MHV-68, and were not detectable in the spleen. Thus, the M4 gene product, in the context of the MHV-76 genome prevented the establishment of detectable latent infection in the spleen.

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Abbreviations

A	Adenosine
AHV-1	Alcelaphine herpesvirus 1
AIDS	Acquired immunodeficiency syndrome
Amp	Ampicillin
AP	Alkaline phosphatase
APC	Antigen presenting cell
Asn-X-Ser/Thr	Asparigine-X-Serine/Threonine
ATG	Initiating methionine
ATP	Adenosine triphosphate
dATP	Deoxyadenosine triphosphate
BAC	Bacterial artificial chromosome
BART	*Bam*HIA rightward transcripts
BCBL	Body cavity based lymphoma
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BHK	Baby hamster kidney
BL	Burkitt's lymphoma
bp	Base pair
BSA	Bovine serum albumin
C	Cytosine
CaPV	Capripox virus
CPV	Cowpox virus
CCR	Chemokine receptor
CCV	Channel catfish virus
CXCR	CXC chemokine receptor
cDNA	Complementary DNA
cm	Centimetre
CMV	Cytomegalovirus
CO_2	Carbon dioxide

CSF	Colony stimulating factor
CTL	Cytotoxic T lymphocyte
dCTP	Deoxycytidine triphosphate
°C	Degrees Celsius
dH_2O	Distilled water
dNTP	Deoxynucleoside triphosphate
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DTH	Delayed type hypersensitivity
DTT	Dithiothreitol
EBER	Epstein-Barr virus early RNA
EBNA	Epstein Barr virus nuclear antigen
EBV	Epstein-Barr virus
E.coli	*Escherichia coli*
EDTA	Ethylene diaminetetraacetic acid
EHV-2	Equine herpesvirus 2
EV	Ectromelia virus
ER	Endoplasmic reticulum
FCS	Foetal calf serum
FITC	Fluorescine isothiocyanate
FLICE	FADD homologous ICE/CED-3-like protease
FLIP	FLICE- inhibitory protein
gp	Glycoprotein
GCR	G protein-coupled receptor
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage-colony stimulating factor
GMEM	Glasgow's modified Eagle's medium
GRO-α	Growth-related oncogene α
GST	Glutathione S-transferase
dGTP	Deoxyguanosine triphosphate

HA	Haemagglutinin
HCl	Hydrogen chloride
HCMV	Human cytomegalovirus
HD	Hodgkin's disease
HEPES	N'-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
His	Histidine
HHV	Human herpesvirus
hr	Hour
HSV	Herpes simplex virus
HVS	Herpesvirus saimiri
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
IM	Infectious mononucleosis
IP-10	Interferon-induced protein-10
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	Kilobase
kD	Kilodalton
KI	Knock in
KO	Knock out
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma-associated herpesvirus
LANA	Latency associated nuclear antigen
LB	Luria Bertani medium
LCL	Lymphoblastoid cell line
LMP	Latent membrane protein
LP	Leader protein
LPD	Lymphoproliferative disease
LUR	Long unique region
M	Molar

MACS `	Magnetic associated cell sorting
MCD	Multicentric Castleman's disease
MCF	Malignant catarrhal fever
MCMV	Murine cytomegalovirus
MCP	Monocyte chemoattractant protein
MCS	Multiple cloning site
MCV	Molluscum Contagiosum
MDV	Marek's disease virus
ME	β-mercaptoethanol
MHC	Major histocompatibility complex
MHV	Murine gammaherpesvirus
MIP	Macrophage inhibitory protein
MLN	Mediastinal lymph node
MM	Millimetre
mM	millimolar
MOI	Multiplicity of Infection
MoMuLV	Moloney murine leukaemia virus
MOPS	Morpholinepropanesulphonic acid
mRNA	Messenger RNA
MV	Myxoma virus
NAP	Neutrophil activating peptide
NBT	Nitro blue tetrazolium
neo	neomycin
NK	Natural killer
nm	nanometre
NPC	Nasopharyngeal carcinoma
OD	Optical density
OHV	Ovine herpesvirus
Oligo(dT)	Oligodeoxythymidine
ORF	Open reading frame

ori	origin of replication
OV	orf virus
p	Prefix for plasmid DNA
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEC	Peritoneal exudate cell
PEG	Polyethylene glycol
PEL	Primary effusion lymphoma
p.i.	Post-infection
pfu	Plaque forming unit
PMSF	Phenylmethylsulphonyl fluoride
PTL	Post-transplant lymphoma
RACE	Rapid amplification of cDNA ends
RANTES	Regulated upon activation, normal T-cell expressed & secreted
RCV	Recombinant virus
RNA	Ribonucleic acid
RPMI	Rosewell Park Memorial Institute
RPV	Rabbitpoxvirus
RRV	Rhesus monkey rhadinovirus
RT	Room temperature
RT-PCR	Reverse transcription PCR
SCID	Severely combined immunodeficient
SDS	Sodium dodecyl sulphate
sec	Second
SFV	Shope fibroma virus
SPV	Swinepox virus
SSC	Standard saline citrate
STP	Saimiri-associated protein
SV40	Simian vacuolating virus 40

T	Thymine
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TEMED	N, N, N', N'-tetraethylmethylenediamine
TFB	Transformation buffer
TH	T helper
TIP	Tyrosine kinase-interacting protein
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TPA	12-O-tetradecanoyl phorbol-13 acetate
TR	Terminal repeat
Tris-HCl	Tris Hydrochloride
TTP	Thymidine triphosphate
TUNEL	Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labelling
UV	Ultraviolet (light)
VaV	Variola virus
VEGF	Vascular endothelial growth factor
v/v	Volume per volume
VV	Vaccinia virus
VZV	Varicella zoster virus
WT	Wild type
w/v	Weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
ZEBRA	Z fragment EBV reactivation antigen

Chapter One:

Introduction

1.1 Herpesviruses

Herpesviruses are complex, enveloped viruses which contain a large double-stranded DNA genome. Analysis of most vertebrates has yielded at least one herpesvirus (Roizman *et al.,* 1992). Hosts include lower vertebrates such as bony fish, reptiles and amphibians, and higher vertebrates such as birds and mammals. Each herpesvirus is usually limited to a single species in natural infections (Minson *et al.,* 1989). This high degree of host specificity implies that the members of the herpesvirus family, of which there are over 100, have evolved in close association with their hosts.

1.1.1 Morphology

The architecture of a typical member of the herpesvirus family consists of a core which contains viral DNA which is present as a toroid (Nazerian, 1974). The virion possesses an icosadeltahedral capsid which is 100-110 nm in diameter containing 162 capsomeres. A hole runs from the surface along the long axis and is 4 nm in diameter (Wildy *et al.,* 1963). The tegument is the term given to an asymmetric structure between the capsid and envelope. Variation in the thickness of the tegument is usually determined by where the virion is situated in the infected cell. This alterable thickness of the tegument contributes in the size of herpesvirions which can range from 120 to 300 nm.

1.1.2 Classification

The original classification of herpesviruses was based on biological properties, but current classification takes into consideration gene content and sequence similarities (Roizman *et al.,* 1981, 1992). The members of the family Herpesviridae are classified into three subfamilies; the Alpha-, Beta-, and Gamma-herpesvirinae.

The Alphaherpesvirinae have a variable host range, grow relatively quickly in cell culture, have a short reproductive cycle, and have the ability to establish latent infections in nervous tissue. VZV (Varicella zoster virus), HSV-1 (herpes simplex virus type 1) and HSV-2 (herpes simplex virus type 2) among others belong to the Alphaherpesvirinae.

The Betaherpesvirinae have a much more restricted host range, exhibit longer reproductive cycles in cell culture, spread more slowly in cell culture and frequently cause enlargement of infected cells. They include CMV (cytomegalovirus), HHV-6 (human herpesvirus type 6), and HHV-7 (human herpesvirus type 7). Viruses in this subfamily are latent in tissues such as lymphoreticular cells, secretory glands and kidneys.

The Gammaherpesvirinae have a host range that is commonly limited to the family which the natural host is a member of. However, there are exceptions to the rule. Members of this subfamily, such as EBV (Epstein-Barr virus), HVS (herpes virus saimiri), MHV-68 (murine gammaherpesvirus-68), AHV-1 (alcelaphine herpesvirus 1) and the more recently discovered KSHV (Kaposi's sarcoma-associated herpesvirus) (Chang *et al.,* 1994) are lymphotropic and tend to be specific for either T or B lymphocytes. The gammaherpesvirus latent form is frequently maintained in lymphoid tissue.

The three herpesvirus subfamilies have been subdivided into genera. The Alphaherpesvirinae have currently been separated into two genera; the genus Simplexvirus which includes HSV-1, and the genus Varicellovirus which includes VZV. The Betaherpesvirinae have currently been separated into three genera; the genus Cytomegalovirus which includes HCMV, the genus Muromegalovirus which includes murine cytomegalovirus, and the genus Roseolovirus which include HHV-6. The Gammaherpesvirinae have been separated into two genera; the genus Lymphocryptovirus (gamma-1 herpesvirus) which includes EBV, and the genus Rhadinovirus (gamma-2 herpesvirus) which includes HVS, KSHV, AHV-1 and MHV-68.

An example of a herpesvirus which does not fit into the criteria of recognised subfamilies is channel catfish virus (CCV). This virus is classified in the

Alphaherpesvirinae. However , sequence analysis has not shown any significant genetic relationship between CCV and alphaherpesviruses (Davison, 1992).

There are four common properties of herpesviruses. Firstly, enzymes such as DNA polymerase and helicase which are involved in nucleic acid synthesis, and thymidylate synthetase and thymidine kinase which are involved in DNA metabolism are produced by all herpesviruses. Secondly, the nucleus is the compartment where viral DNA synthesis and capsid assembly occurs. The capsid is always enveloped as it travels through the nuclear membrane. Thirdly, the release of infectious progeny virus always results in the death of the infected host cell. Lastly, herpesvirus genomes can remain latent in their natural host cells. In latently infected cells, the viral genomes take the form of closed circular molecules and there is a reduction in viral gene expression (Roizman *et al.,* 1992).

1.1.3 Conserved Genes

Herpesviruses possess linear double-stranded DNA genomes ranging from 125 to 230kb and contain 70 to 200 genes. The nucleotide composition varies from 32% to 74% G + C. The unique DNA of herpesvirus genomes contains reading frames which are closely arranged and frequently overlap. There are genes orientated left and right and some which contain splice sites. A widely differing pattern of repeated regions are commonly found either within or at the ends of the genome (McGeoch, 1989, 1992, Roizman *et al.,* 1992).

Overall, the three subfamilies share about 40 conserved or core genes in common as judged by their amino acid sequence comparisons. This strongly implies a common evolutionary origin in that core genes are present as blocks which have been subjected to rearrangement throughout the process of evolution (Davison *et al.,* 1987, Chee *et al.,* 1990, Albrecht *et al.,* 1992). Core genes are present in central processes such as nucleotide metabolism, DNA replication, capsid structure, DNA packaging and virion morphogenesis. The UL50 gene of HSV-1, for example, is a core gene which functions

as a deoxyuridine triphosphatase (McGeoch *et al.,* 1994). This gene is non-essential, that is, UL50 may be removed from the HSV-1 genome without affecting viral growth in cell culture (McGeoch *et al.,* 1994). Core genes do not necessarily correspond to essential genes, however genes non-essential in tissue culture may be essential *in vivo* e.g. UL39 and US2 (Roizman & Sears, 1996).

1.1.4 Unique Genes

Each individual herpesvirus encodes unique genes which have evolved to confer a particular advantage to the virus. The unique genes serve as characteristics of particular subfamilies but some genes are exclusive to a member of a subfamily. These genes are responsible for aspects of the survival of each subfamily such as pathogenesis, latency and immune evasion (Virgin *et al.*, 1997, Albrecht *et al.*, 1992, Russo *et al.*, 1996, Baer *et al.*, 1984).

1.1.5 Gene Expression

In cells productively infected by a herpesvirus, viral gene expression occurs in a sequential order in a cascade fashion. Groups of viral genes which are expressed are termed immediate-early, early or late depending on when their encoded protein is produced post-infection (Honess & Roizman, 1974). Immediate-early genes are defined by their transcription after infection prior to viral protein synthesis, for example, the ICP4 protein of HSV. Early genes are transcribed prior to DNA synthesis and their products may be required to facilitate replication of the viral genome, for example thymidine kinase and DNA polymerase. Late genes encode proteins produced after DNA synthesis is initiated, for example structural proteins such as capsid proteins (Roizman & Sears, 1996). Viral gene expression during latency occurs at a much lower level than during lytic infection. In HSV, LATs (latency-associated transcripts) are present abundantly in latently-infected neurons (Stevens *et al.*, 1988, Krause *et al.*, 1988). During EBV latent infection, at least 11 EBV proteins are expressed (see section 1.2.3). Two genes encode Epstein-Barr virus early RNA (EBER 1 and EBER2), six encode nuclear proteins (EBNAs 1, 2, 3a, b, c, and LP), and three encode integral

membrane proteins (LMP 1, 2A, and 2B) (Kieff, 1996).

1.1.6 Lytic Replication

HSV is the most intensely studied of all herpesviruses and the large amount of information known about herpesvirus lytic replication is predominantly from the HSV work.

Initially, the virus enters a cell via attachment to cell surface receptors mediated by a glycoprotein attachment pathway, resulting in a rapid fusion of the viral envelope with the plasma membrane. The viral DNA is trafficked to the pores of the nucleus by its surrounding viral capsid where it is released into the nucleus and immediately circularises. The nucleus is the site where DNA synthesis proceeds. Sequential expression of the cascade of immediate early, early and late genes ensues (described in section 1.1.5). The virus encodes a number of proteins necessary for DNA synthesis including the DNA polymerase. Replication occurs via a rolling circle mechanism generating head to tail concatemers of unit length viral DNA which are cleaved and packaged into preassembled capsids (Jacob & Roizman, 1977, Jacob *et al.*, 1979). The capsids containing the viral DNA acquire a new protein while viral glycoproteins and tegument proteins accumulate in cellular membranes which attach onto the capsids. It is uncertain how the virus acquires its final envelope membrane but it has been proposed that after intranuclear assembly, the capsids acquire an envelope by budding through the inner lamella of the nuclear membrane. The complete virion then passes through the secretory pathway, and membrane glycoproteins are modified by the endoplasmic reticulum (ER) and Golgi compartments (Johnson & Spear, 1982). An alternative hypothesis suggests a two-step envelopment process in which the virus may acquire the final envelope at the plasma membrane where glycoproteins have been trafficked to. Recent mounting evidence strongly favours the two-step envelopment process. The virus undergoes de-envelopment and then re-envelopment at a post-ER compartment (Browne *et al.*, 1996, Whiteley *et al.*, 1999, Roller *et al.*, 2000, Klupp *et al.*, 2000). Subsequently, the virus buds through the nuclear membrane resulting in an infectious

mature virion (Roizman & Sears, 1996).

1.1.7 Diseases Associated with Alphaherpesviruses

Infection of an individual host for the first time by a herpesvirus can either be symptomatic, or more commonly, clinically inapparent. Primary HSV infection involves virus entry into sensory nerve endings and axonal transport to the neuronal cell body where the latent infection is established. The majority of the adult population is latently infected with HSV but most individuals appear asymptomatic. Individuals that exhibit signs of primary HSV-1 infection present a condition termed acute gingivostomatitis. Reactivation of HSV-1 which occur in a minority of infected individuals result in recurrent lesions, termed 'cold sores'. HSV-2 primary infection can cause aseptic meningtis and extragenital lesions, however, as with HSV-1, many primary genital infections are inapparent. Secondary reactivating symptoms of HSV-2 present as genital lesions. Other diseases associated with HSV are infections of the eye which can cause keratoconjuctivitis and invasion of the central nervous system which presents as herpes encephalitis (Whitley, 1996).

VZV infection of an individual for the first time can cause varicella (chicken pox) and reactivation results in zoster (shingles) (Mahalingam *et al.*, 1999). Varicella occurs in most individuals during their childhood and is usually an uncomplicated disease. However, in immunocompromised patients, varicella is a severe and often life-threatening disease. VZV also establishes a life long latent infection of sensory dorsal root ganglia. Unlike HSV, VZV is disseminated, thus latency is established in multiple ganglia. Zoster is frequent among the elderly. VZV can frequently cause complications, such as post-herpetic neuralgia which involves severe debilitating chronic pain (Arvin, 1996).

1.1.8 Diseases Associated with Betaherpesviruses

CMV infection is acquired at an early age and is common in all populations. Primary infection does not usually cause any symptomatic illness in normal healthy individuals,

but has been associated with a mononucleosis syndrome. However, CMV is a major cause of multi-organ disease in immunocompromised patients. Invasive CMV disease can occur in newborn infants infected in utero, immunocompromised post-transplant patients, and AIDS patients (Britt & Alford, 1996).

Studies with both HHV-6 and HHV-7 have been limited to primary infections of children. Primary HHV-6 infection is the causative agent of exanthem subitum (roseola), a common febrile illness that occurs in early childhood (Pellett & Black, 1996). HHV-7 appears to cause a very similar illness to that reported for HHV-6 (Frenkel & Roffman, 1996). Like CMV, infection of the immunocompromised and AIDS patients can cause severe multi-organ involvement. However, these findings are unclear and remain to be determined.

1.1.9 Diseases Associated with Gammaherpesviruses

Gammaherpesviruses cause significant disease in both humans and domestic cattle. AHV-1 (alcelaphine herpesvirus-1) and OHV-2 (ovine herpesvirus-2) are non-human gammaherpesviruses of economic importance in having a causal association with malignant catarrhal fever (MCF), a fatal lymphoproliferative disorder of domestic ruminants (Bridgen *et al*, 1989, Plowright *et al*, 1960). In humans, EBV the prototypic gammaherpesvirus (see section 1.2) and the more recently discovered KSHV (Kaposi's sarcoma-associated herpesvirus) (see section 1.4) have received the greatest interest due to their association with disease in humans. Herpesvirus saimiri (HVS) has also been well studied due to its oncogenic properties in marmosets and cottontail rabbits (Medvezcky *et al.*, 1989, Wright *et al.*, 1977). Herpesvirus sylvilagus infects cottontail rabbits (Hinze, 1971) and is of interest as this infection resembles infectious mononucleosis in man and has analogy to EBV infection (Medveczky *et al.*, 1984, Kramp *et al.*, 1985).

1.2 Epstein-Barr Virus

In the majority of cases primary EBV infection occurs in childhood and is clinically

inapparent. Over 90% of the human population are asymptomatic carriers and it is generally thought and accepted that EBV is orally transmitted. The individuals who do not encounter the symptom-free primary infection during their childhood undergo delayed primary infection during their adulthood. Half of these cases are asymptomatic whereas the other 50% result in infectious mononucleosis syndrome characterised by lymphadenopathy, particularly in the cervical region. The strong cell mediated immune response that occurs is thought to be the principal acute infection surveillance mechanism (Tomkinson *et al.*, 1987, Lynne *et al.*, 1998).

Virus replication primarily occurs in oropharyngeal epithelial cells and possibly in B lymphocytes in lymphoid-rich areas (Sixbey *et al.*, 1984). After initial infection, virus can frequently be detected in saliva for life (Yao *et al.*, 1985). The major reservoirs for EBV are in the B lymphocytes in the circulating blood and lymphoid and bone marrow tissues (Decker *et al.*, 1996, Khan *et al.*, 1996). Resting B cells harbour EBV in a latent state where the genome is maintained in a non-productively replicating form. Persistent EBV infection is primarily controlled by $CD8^+$ cytotoxic lymphocytes (CTL) as the virus persists in a few circulating B lymphocytes (Murray *et al.*, 1992). Sporadic reactivation of EBV and its infection of cells is effectively controlled by the immune surveillance of viral specific cytotoxic T cells (Moss *et al.*, 1979, Rickinson *et al.*, 1979, Rickinson *et al.*, 1980).

1.2.1 EBV–Associated Diseases

EBV manifests as a disease when the virus-host balance is disturbed and its association with a number of malignancies has generated great interest in the pathogenesis of this gammaherpesvirus. Reactivation of EBV is associated with diseases including Burkitt's lymphoma, Hodgkin's disease, post-transplant lymphoma, T cell lymphomas and nasopharyngeal carcinoma.

EBV emerged as a virus of immense interest when it was identified as the aetiological link with endemic (African) Burkitt's lymphoma (BL) tumour samples (Epstein *et al.*,

1964). Classic BL is B cell tumour found in equatorial African and Papau New Guinea (Burkitt 1970) where it is a predominant childhood malignancy. Over 90% of endemic BLs are EBV positive and though the role of EBV remains unclear, it is generally accepted that the virus is one necessary element in the causation of disease. A sporadic form of BL, with very similar clinical features to endemic BL, occurs with a greatly reduced incidence in other regions of the world with only 20% of tumours containing EBV DNA, although in others this can rise to 80% (Magrath, 1990). Both the endemic and sporadic forms of BL show translocations involving c-myc, an oncogene, which results in its deregulation (Leder *et al.*, 1983, Klein & Klein, 1985, Bornkamm *et al.*, 1987, Rickinson & Kieff, 1996). These studies have implicated the virus in the chain of events which leads to these c-myc translocations.

Organ graft patients who are immunosuppressed to prevent organ rejection are targets for the development of post-transplant lymphomas (PTL) and lymphoproliferative disease. The lymphoproliferative disease can also occur in AIDS patients. All EBV latent genes are expressed in PTL and some AIDS lymphomas, which would suggest that EBV has a significant role in the development of the lesions (Young *et al.*, 1989, Thomas *et al.*, 1990, Gavioli *et al.*, 1993).

EBV DNA has been detected in up to 50% of cases of Hodgkin's disease (HD), a tumour which occurs worldwide. However, evidence is not sufficient to implicate EBV in the aetiology of HD (Weiss *et al.*, 1989, Herbst & Niedobitek, 1993, Rickinson & Kieff, 1996). The condition is characterised by the presence of malignant multinucleate Reed-Sternberg and mononucleate Hodgkin cells within the affected lymph node. Mutations identified in cases of HD are limited but they have been observed in the tumour suppressor gene p53 (Gupta *et al.*, 1993, Niedobitek *et al.*, 1993) and retinoblastoma protein (Morente *et al.*, 1997), indicating an involvement of these genes in the development of HD.

Different types of T cell lymphomas have been linked to EBV infection with a wide

range of incidences, including angioimmunoblastic lymphadenopathy (Ott *et al.*, 1992), nasal lymphoma (Harabuchi *et al.*, 1990) and peripheral T cell lymphoma (Jones *et al.*, 1988). Like many EBV-associated diseases, the role of the virus in the lesions is unclear.

Nasopharyngeal carcinoma (NPC) is a malignancy which occurs rarely throughout the world but is the major cause of death from cancer in southern China (Simons *et al.*, 1974). The tumour develops in the squamous epithelium in the posterior nasopharynx (Raab-Traub, 1993). Studies have indicated the association of EBV with NPC and the presence of the virus DNA in the malignant epithelial cells (Wolf *et al.*, 1973). Environmental co-factors, such as phorbol esters and nitrosamines (tumour promoting chemicals) detected in Chinese salted fish have been linked to areas of high incidence of NPC (Poirier *et al.*, 1987).

Other EBV-associated malignancies include oral hairy leucoplakia in HIV carriers (Greenspan at al., 1985) and extremely rare familial X-linked lymphoproliferative disease (Sayos *et al.*, 1998, Howie *et al.*, 2000).

1.2.2 EBV Genome

The prototypical gamma-1 herpesvirus EBV occurs in over 90% of the world's population. The discovery of EBV as the possible causative virus in BL tumour samples led to the cloning (Dambaugh *et al.*, 1980) and sequencing (Baer *et al.*, 1984) of EBV, the first of any herpesvirus. The EBV genome is a linear double-stranded DNA of 172kb in length with a GC content of 60%. Long terminal repeats (LTRs) flank the unique sequence which contains several internal repeats dispersed across the DNA. To date, only two types of EBV have been defined, type 1 and type 2, also called type A and type B, which have 70-85% DNA sequence homology (Sample *et.al.*, 1990). The differences in their genomes are primarily in the areas which encode the latency associated nuclear proteins. EBV type 1 is more prevalent in western societies whereas both types are isolated with an equal frequency in African countries (Zimber *et al.*,

1986). Type 1 is able to transform cells better than type 2 which has been attributed to variations in two latency associated genes (Rickinson *et al.*, 1987, Rowe *et al.*, 1989).

EBV unique genes and gammaherpesvirus specific genes are among the many open reading frames (ORFs), of which there are over 90, encoded by the EBV genome. The latency associated proteins (described in section 1.2.3) are unique to EBV as no homologues have been described for any other herpesviruses.

A few examples of gammaherpesvirus specific genes encoded by the EBV genome are as follows. A bcl-2 homologue is functionally active as an expressed early gene (Henderson *et al.*, 1993, Tarodi *et al.*, 1994). The EBV R transactivator is expressed as a immediate early gene responsible for regulating the expression of other lytically expressed viral proteins (Ragoczy & Miller, 1999). ZEBRA (Z fragment EBV reactivation antigen) is an immediate early gene associated with reactivation from latency acting as a key transactivator for lytic virus replication (Packham *et al.*, 1990). A homologue of ZEBRA has been identified in KSHV.

Like many gammaherpesviruses, EBV encodes homologues of cellular genes, for example the bcl-2 homologue and the interleukin-10 homologue, which is described later in section 1.6.4.

The EBV genome encodes many glycoproteins including the important glycoprotein, gp340/220 which mediates the virus attachment to the B cell surface by binding to CR2/CD21 (Nemerow *et al.*, 1987, Fingeroth *et al.*, 1984). Binding of EBV to CR2/CD21 on resting B cells results in B cell activation and proliferation (Sinclair *et al.*, 1994).

1.2.3 EBV Latency Genes

The establishment of latency in lymphocytes is critical for the survival and maintenance of gammaherpesviruses within their host. Genes have been identified which are

expressed during EBV latency. These include the six EBV nuclear antigens (EBNAs) termed EBNA 1, 2, 3A, 3B, 3C, LP (leader protein), three latent membrane proteins (LMP) termed LMP 1, 2A and 2B, two small EBV-encoded RNAs termed EBERs 1 and 2, and the BARTs (Kieff, 1996).

EBNA 1 is a DNA binding protein which binds sites in the latent viral origin of replication. EBNA 1 is the only viral protein required for the maintenance of the viral episome, the latent state of the genome (Yates *et al.*, 1985). This latency protein also binds non-specifically to DNA and is involved in the co-ordination of virus genome replication with cell division. BYRF1 encodes the EBNA 2 protein, a transcription factor which activates latent viral genes and cellular genes necessary for the immortalisation of B lymphocytes *in vitro* (Cohen *et al.*, 1989). EBNAs 3A, 3B and 3C are encoded by BERF. Little is known about the three proteins, except that EBNA 3A and 3C are required for B cell immortalisation (Tomkinson *et al.*, 1993). The function of EBNA LP, encoded by BWRF1, is unknown but there have been indications it may bind to p53 and the retinoblastoma gene product which would propose it to be involved in the regulation of cell proliferation (Szekely *et al.*, 1993, Hammerschmidt & Sugden, 1989).

LMP 1 is a viral oncoprotein coded for by the BNLF1 ORF and is the most abundant viral transcript present in latently infected B cells. The LMP 1 protein functions as a cytoplasmic and membrane protein and is essential for B cell immortalisation (Kaye *et al.*, 1993). Recently, LMP 1 has been shown to exert immunosuppressive effects on T lymphocytes *in vivo* and inhibit, as well as stimulate, gene expression (Dukers *et al.*, 2000, Sandberg *et al.*, 2000). Furthermore, LMP-1 has been demonstrated to induce the transporter associated with antigen processing 2 (TAP-2) gene which is associated with and requires the expression of the interferon regulatory factor 7 (IRF-7) (Rowe *et al.*, 1995, Zhang & Pagano, 2001). The LMP 2A and 2B ORFs are situated at opposite ends of the linear EBV genome in BARF1/BNRF1, and are only in entirety when an episome is formed (Laux *et al.*, 1988). Alternative splicing produces the two membrane proteins

which are not required for B cell immortalisation. Little is known about their function. LMP 2A may be involved in the inhibition of calcium mobilisation which is a process associated with the viral lytic cycle and B cell activation (Miller *et al.*, 1994, Crawford & Ando, 1986).

EBERs 1 and 2 are coded for by BCRF1 and are transcribed but not translated. Their RNAs are the most abundant in latently infected cells. The significance of the EBERs is unknown. They are not required for immortalisation of B lymphocytes or for virus replication but may be involved in the regulation of the protein kinase PKR. Recently, the EBERs have been shown to contribute to the tumourigenic potential of BL cells independent of a direct effect on apoptosis (Ruf *et al.*, 2000).

BARTs are a complex family of transcripts of unknown function which are transcribed from the BamHI A region of the genome (Brooks *et al.*, 1993, Smith *et al.*, 2000a).

1.2.4 EBV Latency Types

Three distinct types of latent EBV infection exist, termed I, II and III. The decision where one cell type chooses one latency state over another is not fully understood. *In vitro* studies have demonstrated it is possible to switch from one form of latency to another, which indicates a transcriptional control mechanism may exist (Kerr *et al.*, 1992, Rowe *et al.*, 1992).

The type III model of latent EBV infection is provided by *in vitro* transformed lymphoblastoid cell lines (LCL). The full array of latency genes are transcribed which are the six EBNAs, the three LMP, the two EBERs and the BARTs. This type of expression is also observed in infectious mononucleosis, EBV positive lymphomas of transplant patients, and naïve B cells of healthy tonsils (Tierney *et al.*, 1994, Laytragoon-Lewin *et al.*, 1997, Joseph *et al.*, 2000).

Latency type I was initially observed in EBV-positive Burkitt's lymphoma biopsies.

This type of latency only expresses EBNA1, the EBERs and the BARTs (Rowe *et al.*, 1987, Brooks *et al.*, 1993).

Nasopharyngeal carcinoma cells were the source of the first observations of latency type II (Deacon *et al.*, 1993, Brooks *et al.*, 1992, Hamilton-Dutoit *et al.*, 1993, Young *et al.*, 1988). This type of latency is also seen in Hodgkin's disease. Latency type II gene expression includes EBNA1, the EBERs, the BARTs, LMP1 and/or LMP2A and/or LMP2B (Hitt *et al.*, 1989, Gilligan *et al.*, 1991).

1.3 Herpesvirus Saimiri

Herpesvirus saimiri (HVS) is a T lymphotropic gamma-2 herpesvirus which causes an asymptomatic infection in its natural host the squirrel monkey (*Saimiri sciureus*). However, in New World primates and cottontail rabbits, HVS infection results in rapidly fatal T cell lymphomas (Wright *et al.*, 1977, Medveczky *et al.*, 1989). Transformation of simian (Desrosiers *et al.*, 1986) and human (Biesinger *et al.*, 1990) T lymphocytes *in vitro* can also occur due to infection with HVS. The level of oncogenicity and transforming ability of HVS is accounted for by sequence divergence in the left end of the long unique region of the viral genome and is the basis for classification of HVS into subgroups A, B and C (Desrosiers *et al.*, 1985, Medveczky *et al.*, 1984, Desrosiers *et al.*, 1986, Szomolanyi *et al.*, 1987, Murthy *et al.*, 1989, Biesinger *et al.*, 1990). Two genes termed saimiri-associated protein (STP) and tyrosine kinase-interacting protein (TIP) which reside in the variable left hand end of the HVS genome have been shown to be necessary for oncogenesis (Duboise *et al.*, 1998).

1.3.1 HVS Genome

The HVS genome consists of 112.9kb of unique DNA of low GC content (34.5%) flanked by 1,444bp multiple repeats of high GC content (70.8%) (Albrecht *et al.*, 1992). There are 76 ORFs encoded within the genome, 60 of which are homologous to genes in other herpesviruses. Many cellular homologues are present which include D-type cyclin, G protein-coupled receptor (Nicholas *et al.*, 1992), dihydrofolate reductase

(Trimble *et al.*, 1988), thymidylate synthase (Bodemer *et al.*, 1986), complement control proteins (Albrecht *et al.*, 1992b) and interleukin-17 (see section 1.6) (Spriggs, 1994).

1.4 Kaposi's Sarcoma-Associated Herpesvirus

KSHV (Kaposi's sarcoma-associated herpesvirus) is the most recently discovered human herpesvirus (Ganem, 1997). This gamma-2 herpesvirus was first identified in a Kaposi's sarcoma (KS) tumour of an individual infected with AIDS (Chang *et al.*, 1994). The discovery of the eighth human herpesvirus has led to its implication in the pathogenesis of KS, a vascular tumour consisting of bundles of spindle cells which produce proinflammatory and angiogenic factors. The KSHV genome has also been detected in primary effusion B cell lymphomas (PEL) and multicentric Castleman's disease (MCD) (Mesri *et al.*, 1996; Renne *et al.*, 1996a; Russo *et al.*, 1996; Boshoff *et al.*, 1997, Cesarman *et al.*, 1995a).

In contrast to other herpesviruses, this virus has a unusual distribution in the human population. KSHV appears to have a more limited presence among the human population appearing in clusters of well defined populations. Classic KS in elderly Mediterranean men is usually indolent, human immunodeficiency virus (HIV)-negative endemic KS occurs in certain African populations, HIV-associated KS is the leading neoplasm of AIDS patients, and post-transplant KS has also been observed (Beral, 1991). Epidemiological studies support the theory that KSHV is predominantly sexually transmitted (Gao *et al.*, 1996, Kedes *et al.*, 1996, Blackbourn *et al.*, 1999). A high prevalence occurs in gay and bisexual men with AIDS, a much lower frequency occurs in individuals infected with HIV by heterosexual contact, and it rarely occurs in those infected by blood donations or drug use (Beral *et al.*, 1991). The incidence of childhood KS has also led to the possibility of vertical transmission of KSHV (Ziegler & Katongole-Mbidde, 1996). Saliva has also been observed to harbour KSHV and may be involved in horizontal KSHV transmission (Blackbourn *et al.*, 1998).

KS lesions are composed of endothelial cells and endothelial-like spindle cells (Boshoff

et al., 1995, Staskus *et al.*, 1997). In contrast to conventional cancers, KS is unusual in that the lesions appear to be multifocal and appear to respond to extracellular growth regulatory signals (Nakamura *et al.*, 1988). Compared to the general population, AIDS patients have a 20,000 times higher risk of KS which may be related to the growth factors released from HIV-infected cells in addition to immunosupression (Ensoli *et al.*, 1990). Culturing of KSHV *in vitro* has proven to be difficult with very limited replication achieved in fibroblastoid cells (Foreman, *et al.*, 1997, Renne *et al.*, 1996b, 1998). Like other gammaherpesviruses, KSHV establishes a latent infection characterised by the expression of specific genes and the presence of the episomal form of the genome (Russo *et al.*, 1996, Renne *et al.*, 1996a, Decker *et al.*, 1996, Sarid *et al.*, 1998, Staskus *et al.*, 1997, Zhong *et al.*, 1996, Dupin *et al.*, 1999). B cell lines have been obtained from PEL patients which harbour KSHV in a latent state (Cesarman *et al.*, 1995b, Renne *et al.*, 1996b).

1.4.1 KSHV Genome

Sequence analysis has been performed on a PEL cell line (Russo *et al.*, 1996) and a KS biopsy (Neipel *et al.*, 1997) which has revealed that an identical KSHV sequence is present in both pathologies. The KSHV genome consists of a 140.5kbp long unique region (LUR) flanked by multiple GC rich terminal repeats of 801bp. KSHV is more closely related to HVS, as of the 81 ORFs the LUR encodes, 66 contain sequence similarity to HVS ORFs (Russo *et al.*, 1996).

A number of cellular genes have been pirated by KSHV including the D-type cyclin, bcl-2 and FLICE inhibitor proteins (FLIP). The KSHV encoded D-type cyclin binds to its activating cellular partner cyclin dependent kinase 6 (cdk6) (Godden-Kent *et al.*, 1997), a process not inhibited by normal cell cycle control mechanisms involving cdk inhibitors (Swanton *et al.*, 1997). This allows the viral cyclin to drive the cell through checkpoints ensuring proliferation is achieved. The viral bcl-2 homologue can block apoptosis as efficiently as its cellular counterpart which suggests it is present to ensure survival and proliferation of cells which would otherwise succumb to cell death (Cheng

et al., 1997, Sarid *et al.*, 1997). The viral FLIPs may be involved in preventing apoptosis in cells induced by death receptors and thereby enhance tumour progression (Thome *et al.*, 1997, Djerbi *et al.*, 1999). Cytokine and chemokine elements, including IL-6, GPCR, MIP-I, MIP-II, and MIP-III (see section 1.6.2 and 1.6.4) are also encoded by the KSHV genome. The presence of regulatory genes is an advantage for the survival of KSHV given that KS is largely a growth factor disorder which further supports the role of KSHV in malignant transformation.

Although many KSHV genes have cellular homologues, only a small number are latently expressed in tumour tissues. The latency genes include kaposin A (ORFK12), vFLIP (ORF71/K13), D-type cyclin (ORF72), LNA-1/LANA1 (ORF 73), and the most recently discovered LNA-2/LANA2 (ORF10.5) (Rainbow *et al.*, 1997, Kellam *et al.*, 1997, Kedes *et al.*, 1997, Dittmer *et al.*, 1998, Sarid *et al.*, 1999, Sadler *et al.*, 1999, Jenner *et al.*, 2001, Rivas *et al.*, 2001).

The main immunogenic latent nuclear antigen (LNA-1) is expressed in all three malignancies associated with KSHV (Dupin *et al.*, 1999). LNA-1 has been shown to secure KSHV DNA to chromatin during mitosis (Ballestas *et al.*, 1999, Cotter *et al.*, 1999), inhibit p53 transcriptional activity (Friborg *et al.*, 1999) and interact with the retinoblastoma protein-E2F pathway (Radkov *et al.*, 2000). This supports a role for the latent genes in combating host antiviral immune reponses and ensuring cell proliferation (Djerbi *et al.*, 1999).

1.5 A Gammaherpesvirus Animal Model

EBV is the prototypic and most widely studied member of the gammaherpesviruses. As previously mentioned, the gammaherpesvirus subfamily also include HVS and KSHV. Thus, this finding has heightened the level of interest in the studies of the pathogenesis of gammaherpesviruses. Our goal is to fully elucidate the function and pathogenesis of gammaherpesviruses in man. However, studies have been hampered by both EBV and KSHV being species specific. *In vivo* experiments are restricted to the clinical

presentation of the infection.

Animal models have been of great importance in studies of herpesvirus pathogenesis. Limited gammaherpesvirus animal models exist which include HVS infection of primates and rabbits, AHV-1 infection of rabbits, EBV infection of Old World primates such as rhesus macaques, marmosets and cotton top tamarins (Moghaddam *et al.*, 1997, Finerty *et al.*, 1992). New World primate infection with EBV does not establish a long-term inapparent infection, an aspect essential for gammaherpesvirus latency studies (Shope *et al.*, 1973). No appropriate animal model exists for KSHV infection. However, herpesviruses related to KSHV have been isolated from rhesus monkeys, macaques, African green monkeys, chimpanzees and gorillas (Desrosiers *et al.*, 1997, Rose *et al.*, 1997, Searles *et al.*, 1999, Alexander *et al.*, 2000, Greensill *et al.*, 2000). Studies of EBV in severely combined immunodeficient (SCID) mice have been performed for therapeutic reasons but these studies do not reflect the true nature of the virus infection in its natural host (Mosier *et al.,* 1988, Rowe *et al.,* 1991, Johannessen & Crawford, 1999). Rabbits are only useful as an animal model for non-human gammaherpesviruses and are quite costly. There are many ethical issues concerned with using primates and they are of limited availability and difficult to maintain. Murine models of both human alphaherpesvirus and betaherpesvirus infection exist, however, none exist for the study of gammaherpesvirus pathogenesis. A murine model is desirable because of the large amount of information available on mouse genetics and immunology, and because of the cost efficiency, breeding ability and ease of maintenance of mouse colonies.

The need for a gammaherpesvirus animal model has been filled by murine gammaherpesvirus 68 (MHV-68), a virus which has comparable genetics and pathogenesis to EBV. Characterisation of the infection of mice with MHV-68 has shown that this is an invaluable model system. This system will be advantageous in the observation of the true nature of gammaherpesvirus infection *in vivo*, the origin of disease and the host response, including prophylactic immune mechanisms (Sunil-

Chandra *et al.,* 1992a, b, 1993, 1994a).

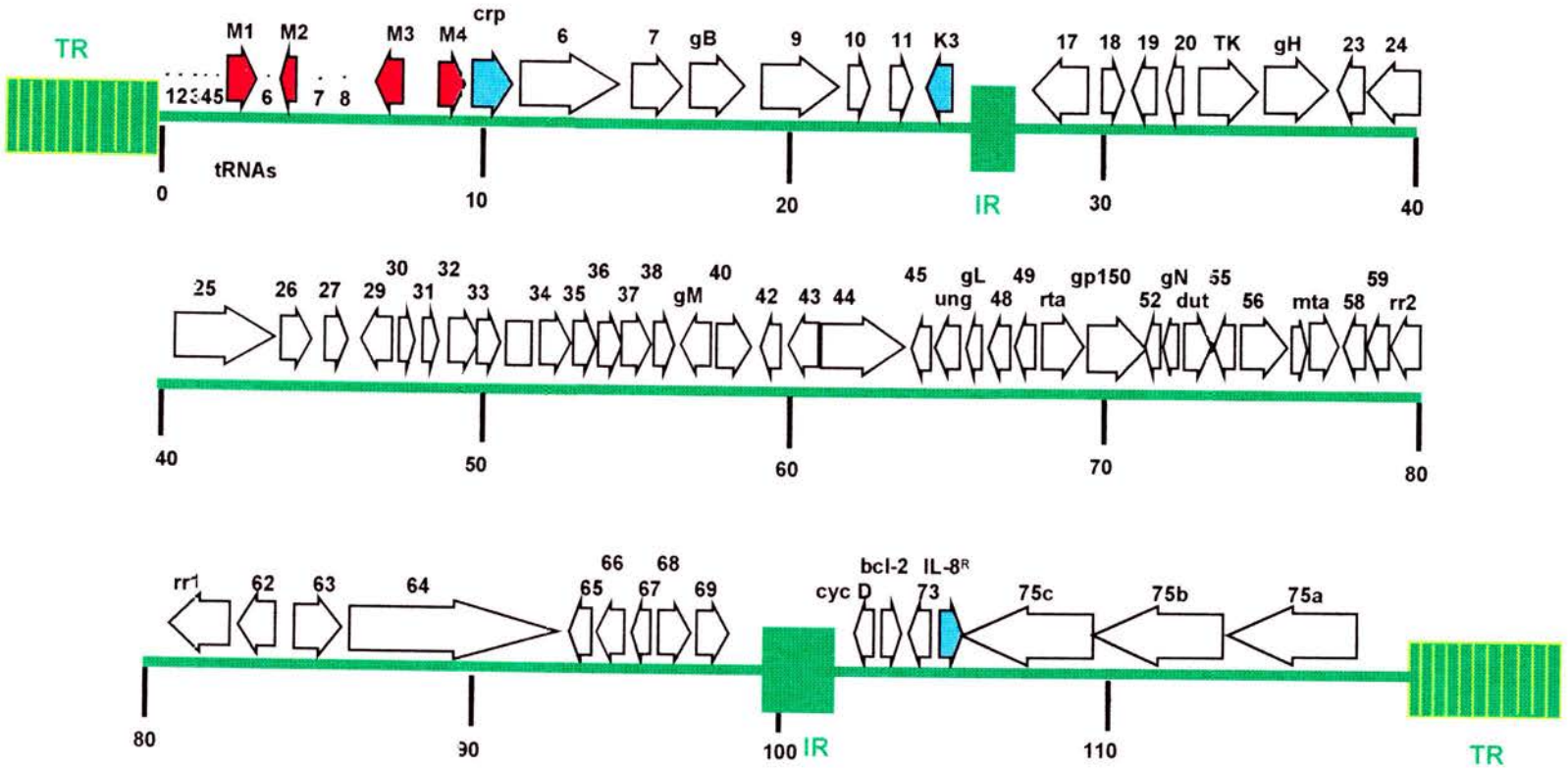
1.5.1 Murine Gammaherpesvirus-68 (MHV-68)

MHV-68 was one of five viruses isolated from free-living murid rodents in Slovakia (Blaskovic *et al.*, 1980). Contrary to EBV, this gammaherpesvirus undergoes a productive infection in standard cell monolayer cultures. The virus is able to infect a large variety of cell types from a number of different species (Ciampor *et al.,* 1981, Svobodova *et al.,* 1982), including Baby Hamster Kidney (BHK) cells which are typically used to quantitate infectious virus using a conventional plaque assay and myeloma cell lines such as NS0's. One cell line termed S11 has been derived from a tumour taken from a MHV-68 infected mouse. This cell line is of B cell origin and all S11 cells are latently infected and bear the genome in a circular or episomal conformation. Of the S11 cells, 2% at any one time undergo spontaneous reactivation to become productively infected. The number of S11 cells undergoing productive infection can be increased to around 10% following the addition of phorbol esters such as TPA (Usherwood *et al.*, 1996b). S11 cells appear to be morphologically similar to EBV-positive lymphoblastoid cell lines (Rickinson & Kieff, 1996) and KSHV-positive B lymphoma lines (Moore *et al.,* 1996).

1.5.2 The MHV-68 Genome

The complete nucleotide sequence of MHV-68 has been determined (Virgin *et al.,* 1997, Davison *et al.,* unpublished results). The MHV-68 genome contains 118, 237 bp of unique DNA of 46% GC content flanked by multiple copies of a 1213 bp terminal repeat of 78% GC content. Within the unique region of the genome there are at least 80 open reading frames (ORFs), many of which are homologues of other gammaherpesvirus genes (see figure 1.5.2). Within those ORFs, 63 have been found to be homologues of HVS genes. In addition, the homologues are present in the KSHV genome and many but not all are in the EBV genome. From comparison of MHV-68 ORFs with their homologues, it has been revealed that this genome is more closely related to HVS and KSHV than to EBV and has therefore been classified as a gamma-2 herpesvirus. Similar

Figure 1.5.2 Murine Gammaherpesvirus 68 genome map



Genomic organisation of MHV-68 (Virgin et al., 1997). The unique region is represented by a single green line with co-ordinates marked off in kilobase pairs. Terminal repeat (TR) elements are shown by green bars with horizontal lines and two internal repeats (IR) are shown by whole green bars. Open reading frames (ORFs) are shown by arrows. ORFs with homologues in other gammaherpesviruses are shown by open arrows and those which are unique to MHV-68 (M1-M4) are highlighted as red arrows. Three genes which may be associated with immunomodulation are shown as blue arrows. The gene number is shown except where the function of the gene is known. crp -complement regulatory protein, gB - glycoprotein B, TK -thymidine kinase, gH - glycoprotein H, gM -glycoprotein M, ung - uracil DNA glycosylase, gL -glycoprotein L, rta -R trancriptional transactivator, gp150 -glycoprotein with homology to EBV gp350, dut - dUTPase, mta -M transcriptional transactivator, rr2 - small subunit ribonucleotide reductase, rr1 -large subunit of ribonucleotide reductase, cyc D - homologue of mammalian cyclin D, bcl-2 - homologue of mammalian bcl-2, IL-8R -homologue of mammalian interleukin-8 receptor.

to other herpesviruses, MHV-68 genes with cellular counterparts have been discovered including a complement regulatory protein, a bcl-2 protein, a D-type cyclin, and a G-protein coupled receptor.

As predicted, the ORF 4 gene has been shown to encode a functional inhibitor of complement activation as measured by inhibition of C3 deposition on activated zymosan particles (Kapadia *et al.*, 1999). Further functions of ORF 4 have been suggested including induction of intracellular signals (Kapadia *et al.*, 1999). Recently an ORF 4 recombinant virus and its corresponding revertant was generated using a system with the MHV-68 genome cloned as an infectious bacterial artificial chromosome (BAC) (Adler *et al.*, 2000). The deletion of ORF 4 revealed that it was not essential for replication of MHV-68 *in vitro*. However, the recombinant virus demonstrated a delay in growth which was further supported by use of the revertant virus (Adler *et al.*, 2000).

Limited homology to bcl-2 family members has been observed with ORF M11. Other viral bcl-2 homologues contain both the BH1 and BH2 domains, which are important for the function of bcl-2 (Kroemer, 1997), and studies have revealed they all have anti-apoptotic activities in vitro (Cheng *et al.*, 1997, Derfuss *et al.*, 1998, Henderson *et al.*, 1993, Kawanishi, 1997, Nava *et al.*, 1997, Sarid *et al.*, 1997, Tarodi *et al.*, 1994). On the contrary, the MHV-68 bcl-2 homologue appears to harbour homology only for the BH1 domain (Virgin *et al.*, 1997) but has still been demonstrated to inhibit Fas- and TNF-induced apoptosis (Wang *et al.*, 1999, Roy *et al.*, 2000) and be expressed during virus persistence (Roy *et al.*, 2000). Unlike the other viral bcl-2 homologues, the MHV-68 bcl-2 homologue has been shown to be susceptible to caspase, an apoptosis-inducing protease. Although the MHV-68 bcl-2 was susceptible its cleavage product functioned similarly to the other unsusceptible viral bcl-2 homologues by preventing proapoptotic activity (Bellows *et al.*, 2000). It would appear that these properties of viral bcl-2 homologues assist virus propagation by facilitating host survival and overcoming programmed cell death in both productively and persistently infected cells.

Similar to KSHV and HVS (Li *et al.*, 1997, Nicholas *et al.*, 1992), a D-type cyclin homologue is predicted to be encoded by ORF 72 of MHV-68 (Virgin *et al.*, 1997). The MHV-68, KSHV and HVS cyclin homologues all share conserved residues, and both KSHV and HVS homologs have been shown to be involved in binding to cyclin-dependent kinase 6 (cdk6) (Godden-Kent *et al.*, 1996, Li *et al.*, 1997, Jung *et al.*, 1994). Studies with the MHV-68 cyclin homologue have demonstrated it has a role in oncogenesis by inducing cell cycle progression in primary lymphocytes and transgenic mice expressing the protein develop lymphoblastic lymphomas with age (van Dyk *et al.*, 1999). A recombinant virus lacking the MHV-68 cyclin homologue has recently been generated showing growth of the cyclin-deletion mutant is efficient *in vitro* and with the same kinetics as the wild type virus (Hoge *et al.*, 2000). However, a replication deficiency occurs upon acute infection in BALB/c mice and the recombinant virus can also establish latency but reactivation is poor. This work was confirmed by the restoration of wild-type phenotype using a revertant virus (Hoge *et al.*, 2000). These studies indicate there may be multiple roles for the MHV-68 D-type cyclin homologue including oncogenesis, acute infection and efficient reactivation from latency.

ORF 74 has homology to genes encoding G-protein coupled receptors (GCR) and may function as an IL-8 receptor. If ORF 74 is found to be a functional IL-8 receptor, it is possible that host cytokines signalling through the viral GCR would affect the pathogenesis of MHV-68. The MHV-68 GCR homologue is one of many gammaherpesvirus GCR homologues discovered (see section 1.6.2). Other immunomodulation genes have also been found and are described in section 1.6.

ORF 50 of MHV-68 is an immediate early gene which has homology, along with HVS and KSHV, to the EBV Rta gene which is also gene 50 encoded. Both ORF 50 genes of KSHV and EBV have been demonstrated to have a significant involvement in the reactivation of latent virus (Lukac *et al.*, 1998, Ragoczy *et al.*, 1998, Sun *et al.*, 1998, Zalani *et al.*, 1996). The MHV-68 encoded rta homolog has been characterised (Liu *et al.*, 2000) and has also been shown to play a central role in viral reactivation from

latency (Wu *et al.*, 2000).

Other MHV-68 ORFs have been found to be homologues of gammaherpesvirus glycoproteins, for example, ORF 8, 22, 39, 47, and M7 are homologues of gB, gH, gM, gL, and gp150 (Efstathiou *et al.*, 1990a, Efstathiou *et al.*, 1990b, Stewart *et al.*, 1994a, Stewart *et al*, 1996, Pepper *et al.*, 1996, Virgin *et al.*, 1997). Studies have only been performed on the MHV-68 gB and gp150 genes (Stewart *et al.*, 1994a, Stewart *et al.*, 1996). Like the EBV-encoded gB, the MHV-68-encoded gB is not expressed on the virion envelope or the cell membrane of infected cells. This is in contrast to the HSV-encoded gB which is essential for initial attachment to a cell surface (Cai *et al.*, 1988, Spear *et al.*, 1992, Shieh *et al.*, 1992, Trybala *et al.*, 1993, Laquerre *et al.*, 1998). The gp150 gene of MHV-68 was selected for study as it is a potential homologue of gp340/220, an abundant glycoprotein present on the surface of virions and a major neutralising determinant of EBV (Nemerow *et al.*, 1987).

1.5.3 MHV-68 Unique Genes

The MHV-68 genome harbours a number of unique ORFs, including M1-M4 and 8 tRNA-like structures (Bowden *et al.,* 1997), that reside in the left hand end of the genome and have no clear homology to genes present in the other gammaherpesviruses. However, in the other gammaherpesviruses, virus-specific genes are located in similar regions and have been shown to be associated with latency or transformation. This has generated great interest in the putative gene functions of the genes at the left hand end of the MHV-68 genome. The unique ORFs are predicted to encode proteins with a minimum of 100 amino acids in length. Studies have shown that this left hand region, along with others, is transcriptionally active during latency resulting in a further heightened interest (Virgin *et al.*, 1999a).

The 8 vtRNAs with significant homology to mammalian tRNAs are highly expressed within splenic germinal centres of latently infected mice and are similar to the EBER genes in that their biological function is unclear. The vtRNAs can act as a marker for

virus latency and are considerably important for detecting latent virus (Bowden *et al.*, 1997, Simas *et al.*, 1999). However, it is important to note that no homologues of the other EBV latency genes have yet been found in MHV-68. The function of these putative tRNA-like genes in viral pathogenesis is unclear as they are not aminoacylated, hence may not have a functional role as tRNAs.

The M1 gene exhibits sequence homology to a poxvirus serine protease inhibitor, SPI-1, the first observation of its kind among the herpesviruses, and homology to the M3 gene, another unique gene of MHV-68 (Virgin *et al.*, 1997, Simas *et al.*, 1998). Poxvirus serpins have been linked to a wide variety of functions including the regulation of host cell range by altering apoptosis (Brooks *et al.*, 1995). However, M1 does not appear to harbour the active site of serpins, therefore it is unlikely to function as a serpin. Studies in which the M1 gene was deleted had no effect on the primary infection or the establishment of latency (Simas *et al.*, 1998). However, disruption of M1 with a *lacZ* expression cassette resulted in enhanced reactivation from latency, proposing M1 may be involved in a pathway which functions to suppress virus reactivation (Clambey *et al.*, 2000).

The M2 gene has an unknown function but studies which have detected M2 viral transcripts in latently infected mice and in latently infected cells *in vitro* have proposed M2 is a latency-associated protein (Husain *et al.*, 1999, Virgin *et al.*, 1999). These studies also identified an epitope in the M2 protein that is recognised by $CD8^+$ T lymphocytes which demonstrated this protein is a target for the host cytotoxic lymphocyte response (Husain *et al.*, 1999). The induction of the $CD8^+$ T cell response to the M2 antigen has been examined and has shown that the antigen-specific T cell response contributes to the control of latently infected cells (Usherwood *et al.*, 2000). This finding provides vital clues and will assist the development of a long-term prevention of gammaherpesvirus diseases.

The M3 gene lacks homology to all known genes including chemokine elements,

however, it has recently been characterised and shown to encode an abundantly secreted protein (van Berkel *et al.*, 1999). Studies by two independent groups have demonstrated the M3 protein binds a number of chemokines, blocks chemokine interactions with cells, and prevents chemokine calcium signalling (Parry *et al.*, 2000, van Berkel *et al.*, 2000). The M3 transcript is transcribed during latency (Simas *et al.*, 1999, Virgin *et al.*, 1999). This finding along with its function as a chemokine binding protein allows us to predict a role for M3 in establishing or reactivating latency and in immune evasion.

1.5.4 The M4 Gene

Apart from the sequence of the M4 gene, there is no information about this novel gene. The start codon of the M4 gene is located 8598bp from the left hand end of the MHV-68 unique region and the predicted coding region is about 1.3kb in length (Virgin *et al.*, 1997). The encoded M4 protein is expected to have a molecular weight of 44.8kD. No studies as yet have been performed to elucidate the function of the M4 protein. Viruses have the propensity to harbour genes with an immunomodulatory role. Thus, it is possible that M4 may function as a viral-encoded cytokine.

1.5.5 MHV-68 Latency-Associated Genes

MHV-68 latent gene expression is unclear due to sensitivity issues with the methods of detection. Studies have been performed which have proposed candidate latency genes of MHV-68 (Simas *et al.*, 1999, Virgin *et al.*, 1999). Candidate latency-associated genes include, ORF M2, ORF M3, ORF M9, ORF M11 (bcl-2 homologue), ORF 72 (cyclin homologue), ORF 73 (KSHV LANA homologue), ORF 74 (GCR homologue) and the 8 tRNAs. Initially, no other genes except the M3 gene was identified as a candidate latency gene using *in situ* hybridisation (Simas *et al.*, 1999). Further studies using nested reverse transcriptase-PCR (RT-PCR) detected the other candidate latency genes in addition to the M3 gene (Virgin *et al.*, 1999). However, possible contamination of the RT-PCR with viral lytic transcripts may have occurred. The M2 protein has been shown to be latency-associated and has an epitope that is recognised by cytotoxic T lymphocytes (Husain *et al.*, 1999). Detection of candidate latency-associated genes in

some cell types and not others have suggested, like EBV, there may be multiple forms of MHV-68 latency (Husain *et al.*, 1999).

None of the EBV latency-associated genes are conserved among the members of the gammaherpesvirus family. In HVS, STP and TIP are transformation-associated gene products which are not conserved in the gammaherpesviruses (Damania *et al.*, 2000). This supports the evidence that MHV-68 virus-specific genes are candidates for viral latency.

1.5.6 Murine Gammaherpesvirus-76 (MHV-76)

MHV-76 is a second murine gammaherpesvirus which was isolated from a wood mouse and recent studies have aroused great interest in this isolate. Sequence analysis of MHV-76 revealed that the left hand end of the genome had been deleted including the M1-M4 genes and the 8 tRNAs. Apart from this deletion, MHV-76 harbours the full contingency of MHV-68 genes. The growth kinetics of MHV-76 are similar to MHV-68 *in vitro* but in contrast it appears to be cleared more efficiently *in vivo* in the lungs of mice (Macrae *et al.*, 2001). Like MHV-68, MHV-76 establishes latency in the spleens of infected mice but the ability to do this is reduced (Macrae *et al.*, 2001). These observations along with the M gene studies (see section 1.5.3) show the left hand end of the genome is associated with the pathogenesis of MHV-68 and in the establishment of latency.

The discovery of MHV-76 has provided us with a useful tool which we can manipulate for molecular biology studies. Studies involving the replacement of each of the individual deleted M genes at the left hand end would be desirable for *in vitro* and *in vivo* studies and would provide an insight into the importance of this region.

1.5.7 MHV-68 Infection and Pathogenesis

Wild rodents are infected by a MHV-68-like virus, however, the mechanism of the route of entry is not known (Blaskovic, 1984). From observation of other gammaherpesvirus

infections, it is probable that the route of infection involves the rodents coming into close proximity with each other, and thereby transmitting the virus via the respiratory pathway.

In experimental studies using inbred mice, intranasal infection is the most common method of infection with MHV-68 as this represents the most likely route of infection in nature. Alternatively, the virus may also be spread through saliva between rodents. Acute infection is established in the lungs and this was demonstrated by laboratory strains of mice which were inoculated intranasally with $4x10^5$ p.f.u. of MHV-68. The initial productive infection in the lung causes an interstitial and peri-bronchiolar pneumonia (Blaskovic *et al.,* 1984; Rajcani *et al.,* 1985; Sunil-Chandra *et al.,* 1992a). This infection involves alveolar epithelial cells and mononuclear cells surrounding airways and blood vessels. Before immune system clearance, this primary infection lasts for around 10 days (Ehtisham *et al.,* 1993). During the period of primary infection, an intense inflammatory infiltrate is observed and this may exist up to 30 days post infection in the form of chronic granulomatous lesions (Sunil-Chandra, 1991).

Prior to the clearance of the productive infection in the lung, MHV-68 has been found to spread to the spleen where the virus is concealed in a latent form in B lymphocytes (Sunil-Chandra *et al,* 1992a, 1992b). In the spleen, infectious virus is rarely detected. Detection of latent virus is based on quantitating reactivating cells in an infective centre assay. This technique has revealed that latently infected cells are present in the first week of an intranasal infection. The level of latently infected cells peaks during the second to third week at numbers of 1 in $10^3/10^4$ splenocytes, before decreasing to steady numbers of 1 in 10^6 spleen leukocytes (Sunil-Chandra *et al.,* 1992a, 1992b). Lower numbers of latently-infected cells are also detected in lymph nodes and bone marrow (Cardin *et al.,* 1996). Latent infection has also been detected in other cell types including peritoneal macrophages (Virgin *et al.*, 1999). As mentioned previously, the virus encoded tRNA-like genes are an invaluable tool by acting as a molecular marker of latency. During productive infection, these tRNA-like genes are expressed but they are

also detected in cells of the germinal centres in spleens in the absence of any productive infection (Bowden *et al.,* 1997). Comparable with all other herpesviruses, latency is a permanent feature in the host.

Contrary to the spleen, virus cannot be detected in lungs later than 10 days post-infection using standard biological assays. However, MHV-68 can persist in the lung indefinitely without any evidence of infection of any other organs such as the spleen. This has been observed using sensitive PCR analysis of virus DNA in B cell deficient mice (Usherwood *et al.,* 1996c). Evidence which indicates that both latent and productively infected cells are present in the lung was achieved by the detection of both linear and circular forms of the genome (Stewart *et al.,* 1998).

It has been a common notion that latently infected lymphocytes are vital for gammaherpesvirus persistence. However, Stewart *et al.,* 1998, have now demonstrated using *in situ* hybridisation for viral tRNAs, alveolar epithelial cells which are latently infected. Hence, epithelial tissue in the lung can act as a non-lymphoid reservoir for gammaherpesvirus persistence and may also be a reservoir for transmission. The lungs are a completely separate site of virus persistence as they do not need to be constantly re-seeded from circulating B cells. MHV-68 latency has also been detected in peritoneal macrophages and dendritic cells (Weck *et al.*, 1996, Weck *et al.*, 1999a, Weck *et al.*, 1999b, Flano *et al.*, 2000). B cells are clearly a principal site of latency and persistence, but are not essential for persistence of the virus which can be harboured in other cellular reservoirs.

Chronic lung infection can be detected in MHC class II deficient mice. These mice restrain the primary infection in the lung but virus is once more observed by day 20 post-infection and exists for up to 3 months. This has been found with EBV replication which has been associated with chronic lung disease in humans (Egan *et al.,* 1995).

In some instances, such as mice that are less than 4 weeks old or immunocompromised,

different tissues can be productively infected by virus, including the heart, liver, kidney, adrenal glands, and the nervous system (Sunil-Chandra, 1991).

1.5.8 Lymphoproliferative Disease

Members of the gammaherpesvirus subfamily are associated with lymphoproliferative disorders. EBV is the etiologic agent of infectious mononucleosis and is also correlated with nasopharyngeal carcinoma and Burkitt's lymphoma. KSHV is implicated in the pathogenesis of KS, a neoplasm frequently detected in AIDS patients (Chang *et al.,* 1994, Mesri *et al.,* 1996, Renne *et al.,* 1996a, Russo *et al.,* 1997, Boshoff *et al.,* 1997, Cesarman *et al.,*1995a).

MHV-68 has been demonstrated to cause chronically infected inbred mice to develop lymphoproliferative disease (LPD) which has clear similarities to the disease seen in EBV-infected individuals. 9% of BALB/c mice infected with MHV-68 over a period of 3 years developed lymphoproliferative disease. Of these, 50% displayed high grade lymphomas. These lymphomas were identified with both lymphoid and non-lymphoid tissues, for example, the liver and kidney. The tumours contained both B and T cells. The B cells were light chain restricted which denoted a clonal origin (Sunil-Chandra *et al.,* 1994a). Cell lines have been derived from mice with LPD, including S11, which is a cell line which originates from B cells and contains virus in the latent episomal form (Usherwood *et al.,* 1996b).

1.5.9 Immunology of MHV-68 Infection

MHV-68 infection of the lung induces a characteristic inflammatory infiltrate consisting of mostly mononuclear phagocytes and lymphocytes (Sunil-Chandra *et al.,* 1992a). Cell infiltration studies by bronchoalveolar lavage reveal that monocytes appeared early and peak around day 3 to 4. Reduction in the number of monocytes occurs as $CD8^+$ T cells are detected which peak at day 8 to 10. $CD4^+$ T cells and B cells are undetectable in the lavage but are seen in lung sections (Sunil-Chandra, NP and Nash, AA, unpublished).

$CD8^+$ T cells present in the lungs are thought to play a central anti-viral role in the control of acute infection (Ehtisham *et al.*, 1993, Stevenson *et al.*, 1998). Absence of $CD8^+$ T cells during the infection in BALB/c mice results in an infection which continues uncontrolled. $CD4^+$ T cells and anti-viral antibody are detected, however, depletion of either or both of these only has a trivial effect on viral clearance (Ehtisham *et al.*, 1993, Usherwood *et al.,* 1996c; Weck *et al.,* 1996, Cardin *et al.*, 1996, Stevenson *et al.*, 1998, Stevenson *et al.*, 1999a). Absence of both T cell subsets led to a severe infection with infection of other tissues as a result of viral dissemination (Ehtisham *et al.*, 1993).

Splenomegaly is correlated with the presence of latently-infected B cells in the spleen during early MHV-68 infection. This was demonstrated in studies with the intranasal infection of transgenic μMT mice that were deficient in mature B cells and therefore have no latent infection in the spleen (Usherwood *et al.,* 1996c; Weck *et al.,* 1996). Consequently, these mice did not exhibit any splenomegaly. B cells are critical for transporting the virus to the spleen (Stewart *et al.*, 1998). There is a role for $CD4^+$ T cells in the induction of splenomegaly as a result of MHV-68 infection. Splenomegaly is observed 14 days post infection where cell numbers double with a $CD4^+$ T cell mediated expansion in B and T cells, $CD8^+$ T cells possessing the greatest elevation. No splenomegaly is observed in studies with CD4 depleted mice (Usherwood *et al.,* 1996a). Furthermore, there is a dependency for $CD4^+$ T cells in the nonspecific B cell activation and polyclonal antibody secretion associated with the viral infection (Stevenson *et al.*, 1999a) and for the production of the virus-specific antibody response (Stevenson *et al.*, 1999b). An infectious mononucleosis-like syndrome is detected after 21 days post infection which is involved with a major expansion of $V\beta4^+$ $CD8^+$ T cells (Tripp *et al.*, 1997, Doherty *et al.*, 1997). $CD4^+$ T cells are important for the expansion of the $V\beta4^+$ $CD8^+$ subset and may be involved in generating the ligand which drives this subset (Flano *et al.*, 1999).

Levels of IL-6, IL-10 and IFN-γ rapidly elevate during splenomegaly which may be

linked with a role for these cytokines in B cell growth and thereby assist viral persistence (Sarawar *et al.,* 1996, Sarawar *et al.*, 1997, Sarawar *et al.*, 1998). Infected B cells stimulate the activation of $CD4^+$ T cells which act as helpers during the proliferation of both infected and non-infected B cells, resulting in B cell expansion. This positive feedback loop, influenced by B cells and $CD4^+$ T cells, creates the splenomegaly.

$CD8^+$ T cells have a central role in the decline of infected B cells and thus in the outcome of splenomegaly. The importance of $CD8^+$ T cells has been demonstrated by depletion studies (Ehtisham *et al.*, 1993, Weck *et al*, 1996, Stevenson *et al.*, 1999b). This proposes a vital role for $CD8^+$ T cells in the regulation of latent B cells. It also suggests that some infected B cells are susceptible to T cell recognition whereas others escape from this effector response resulting in lifetime persistence of the host. During EBV latent B cell infection, T cells target type III latency whereas type I latency evades recognition (Rooney *et al.*, 1985). Different types of latency remains to be elucidated in MHV-68 infected B cells.

Cytokines, such as IFN-γ may have a role in $CD8^+$ T cell control. A study with IFN-γ knockout mice have shown that this lesion had no effect and that IFN-γ is not essential for recovery from acute MHV-68 infection (Sarawar *et al,* 1997). In contrast, another group studying IFN-γ knockout mice infected with MHV-68 showed that nearly one third of these mice died (Kulkarni *et al.,* 1997). There have also been studies using mice with a targeted deletion in the IFN-γ receptor α chain (IFN-γ $R^{-/-}$). These mice still produce IFN-γ but are unable to respond to it. The IFN-γ $R^{-/-}$ mice can control the lung infection but show severe pathological changes in their spleens. This immunopathology is mediated mainly by $CD8^+$ T cells, as the changes are completely reversed in $CD8^+$ T cell-depleted mice (Dutia *et al.,* 1997).

Antibodies against MHV-68 first appear in the serum towards the end of the first week in mice which have been inoculated intranasally (Kulkarni *et al.,* 1997). Mice deficient

in antibody ($\mu MT^{-/-}$ mice) resolve the virus from the lung only slightly slower than wild type mice. However, in the absence of $CD8^+$ and $CD4^+$ cells, the virus reactivates suggesting antibodies are important in preventing reactivation (Stewart *et al.*, 1998).

Studies using mice with a lesion in the α/β interferon receptor gene (IFN-α/β $R^{-/-}$) have demonstrated that type I interferons play a central role in controlling the primary replication of the virus (Kulkarni *et al.*, 1997, Weck *et al.*,1997, Dutia *et al.*, 1999). The absence of type I interferons seems to allow the virus to replicate to immense levels which overcome the immune system of the mice. The IRF-1 gene is a transcriptional transactivator important for the induction of type 1 interferons (Matsuyama *et al.*, 1993, Kimura *et al.*, 1994). Mice that lack the IRF-1 gene ($IRF\text{-}1^{-/-}$) were highly susceptible to MHV-68 infection and behaved similarly to the IFN-α/β $R^{-/-}$mice (Dutia *et al.*, 1999).

1.5.10 Therapeutic Strategies

MHV-68 is an important model for investigating the effects of novel therapeutic strategies against human and animal gammaherpesvirus infection and disease. Immunotherapies include the effects of novel vaccination strategies, antiviral drugs, and candidate CTL vaccines.

Vaccination studies have been performed using the MHV-68 gp150 gene to model the potential of its EBV homolog as an immunogen in protection against EBV (Stewart *et al.*, 1999). Mice were vaccinated with a recombinant vaccinia virus expressing MHV-68 gp150 and then subsequently challenged with a MHV-68 infection. The immunised mice exhibited greatly reduced splenomegaly, comparable to infectious mononucleosis in EBV, but did still establish latency (Stewart *et al.*, 1999).

Thymidine kinase is a protein encoded by EBV that will activate the anti-herpetic drug acyclovir (acycloguanosine) (Pepper *et al.,* 1996). Drug phosphorylation via the thymidine kinase results in the termination of DNA synthesis. Acyclovir also has an effect on MHV-68 productive replication. Another MHV-68 sensitive nucleoside

analogue is 2'-deoxy-5-ethyl-beta-4'-thiouridine (4'-S-EtdU) which has proven to be much more effective (Sunil-Chandra *et al.,* 1993, 1994b, Barnes *et al.*, 1999). This drug delays the onset of latency and rapidly controls infection in the lung. An advantage of these drugs in being only effective against productive replication is that this allows them to be used as an extremely important tool for deciphering the functional events during ganmaherpesvirus latency.

Knowledge of CTL epitopes has led to investigations into the possible use of them at generating protective immunity. During one study, dendritic cells were pulsed *in vitro* with defined lytic cycle MHV-68 CTL epitopes and used to immunise mice (Liu *et al.*, 1999). Subsequent challenge with MHV-68 led to a partial protection against acute infection in the lungs and a reduction in the level of viral latency. Recently in another study, mice were primed with a recombinant vaccinia virus and then boosted by a recombinant influenza virus, both viruses expressing the MHV-68 p56 CTL epitope (Stevenson *et al.*, 1999c). A marked increase of p56 CTL occurred that led to a massive reduction in the acute infection in the lungs. However, neither of the immunisation studies prevented the establishment of latency. These studies did not examine latent infection. Therefore, the discovery of novel CTL epitopes of latent antigens would be an ideal vaccination strategy. As previously mentioned, a candidate latent gene, the M2 gene of MHV-68, has been identified as a target for $CD8^+$ T cells (Husain *et al.*, 1999, Usherwood *et al.*, 2000). M2-specific T cell clones could be used to limit the number of latently infected cells. Studies using this epitope in MHV-68 infection have potential for gammaherpesvirus immunotherapeutic applications.

1.6 Viral Immunomodulatory Proteins

Host immune systems are complex machinery which viruses have coevolved to combat and evade. Large DNA viruses, such as herpesviruses and poxviruses, express proteins that function to either mimic or modulate the host's immune response (Spriggs *et al.*, 1996, Smith, 1997a, Mahalingam & Karupiah, 2000, Alcami & Koszinowski, 2000, Lalani *et al.*, 2000). These viral proteins include chemokine and cytokine elements

which are devoted to their defence strategy. A large proportion are protein receptors and secreted proteins with known cellular homologues. However, an ever increasing number of proteins, in particular viral chemokine-binding proteins and secreted cytokines, exist with as yet unknown homologues (table 1.6a and 1.6b).

Table 1.6a. Viral chemokine homologues, receptor homologues, and binding proteins.

Gene	Virus	Homologue	Mechanisms
Viral chemokine homologues			
vMIP-I/K6	HHV-8	MIP-Iα	CCR8 agonist (Endres *et al.*, 1999).
vMIP-II/K4	HHV-8	MIP-Iα	Broad-spectrum CC, CXC,CX_3C chemokine agonist (Sozzani *et al.*, 1998, Chen *et al.*, 1998, Boshoff *et al.*, 1997).
vMIP-III/BCK	HHV-8	MIP-Iβ	Not determined (Stine *et al.*, 2000).
U83	HHV-6	MIP-Iα	CC chemokine-like agonist (Zou *et al.*, 1999).
MCK-I/m131	MCMV	CC chemokines	CC chemokine-like agonist, promotes viral dissemination (Fleming *et al.*, 1999).
vCXC-I/UL146	HCMV	IL-8	CXC chemokine-like agonist (Penfold *et al.*, 1999).
vCXC-2/UL147	HCMV	IL-8	Not determined (Penfold *et al.*, 1999).
Unmapped	Stealth virus	GRO-α/MGSA	Not determined (Martin *et al.*, 1999).
Unmapped	MDV	IL-8	Not determined (Peng *et al.*, 1995).
vMCC-I/MCI48R	MCV	MCP-I	Broad-spectrum CC and CXC chemokine antagonist (Krathwohl *et al.*, 1997, Damon *et al.*, 1998, Luttichau *et al.*, 2000)
Viral chemokine receptor homologues			
ORF74	HHV-8	CXCR2	Constitutively signalling and agonist-independent receptor (Arvanitakis *et al.*, 1997).
ECRF3/ORF74	HVS	CXCR2	Functional CXC chemokine receptor (Ahuja & Murphy, 1993).
ORF74	MHV-68	CXCR2	*In vitro* transforming activity (Wakeling *et al.*, 2001).
ORF74, E1, E6	EHV-2	CXCR2/CCR1	Not determined (Telford *et al.*, 1995).
U12	HHV-7	CCR	Not determined (Nicholas, 1996).
U12	HHV-6	CCR	Functional CC chemokine receptor (Isegawa, 1998, Milne *et al.*, 2000).
U5	HHV-6	CCR	Not determined (Gompels *et al.*, 1995).
US28	HCMV	CCR1	Functional CC chemokine receptor (Vieira *et al.*, 1998).
UL33	HCMV	CCR1	Localises to viral envelope particles (Margulies *et al.*, 1996).
US27	HCMV	CCR1	Not determined.
UL78	HCMV	CCR1	Not determined.
M33	MCMV	CCR1	Important for viral dissemination to salivary glands (Davis-Poynter *et al.*, 1997).
K2R	SPV	CXCR	Not determined (Massung *et al.*, 1993).
Q2/3L	CaPV	CCR	Not determined (Cao *et al.*, 1995).
Viral chemokine-binding proteins			
M-T7	MV	IFN-γ receptor	Binds C, CC, CXC chemokines via a heparin binding site (Lalani *et al.*, 1997).
M-T1	MV	?	Broad-spectrum chemokine inhibitor (Lalani *et al.*, 1998).
S-T1	SFV	?	Broad-spectrum chemokine inhibitor (Graham *et al.*, 1997).
35kDa	RPV	?	Broad-spectrum chemokine inhibitor (Lalani *et al.*, 1998).
C23L/B29R	VV	?	Broad-spectrum chemokine inhibitor (Smith & Alcami, 2000).
G3R	VaV	?	Broad-spectrum chemokine inhibitor (Smith *et al.*, 1997b).
DIL/H5R	CPV	?	Broad-spectrum chemokine inhibitor (Smith *et al.*, 1997b).
M3	MHV-68	?	Broad-spectrum chemokine inhibitor (Parry *et al.*, 2000).

CCR - chemokine receptor, CXCR - CXC chemokine receptor, GRO-α - growth-related oncogene α, IFN-γ - interferon γ, IL-8 - interleukin-8, MIP-1α - macrophage inflammatory protein 1α, MDV - Marek's disease virus, MCV - Molluscum Contagiosum, SPV - Swinepox virus, CaPV - Capripox virus, MV - myxoma virus, SFV - shope fibroma virus, RPV - rabbitpoxvirus, VV - vaccinia virus, VaV - variola virus, CPV - cowpox virus, HHV - human herpesvirus, MCMV - murine cytomegalovirus, HCMV - human cytomegalovirus, HVS - herpesvirus saimiri, MHV-68 - murine gammaherpesvirus-68.

Table 1.6b. Viral secreted cytokines and cytokine homologues.

Gene	Virus	Homologue	Mechanisms
Viral secreted cytokines			
M-T2	MV, SFV	?	Secreted protein that binds TNF (Upton *et al.*, 1991).
CrmB	CPV, VaV	?	Secreted protein that binds TNF and LT (Alcami *et al.*, 1999).
CrmC	CPV, VV	?	Secreted protein that binds TNF (Alcami *et al.*, 1999).
CrmD	CPV, EV	?	Secreted protein that binds TNF and LT (Alcami *et al.*, 1999).
CrmE	CPV	?	Secreted protein that binds TNF (Alcami *et al.*, 1999).
B15R	VV	?	Secreted protein that binds IL-1β (Alcami & Smith, 1992).
B18R	VV	?	Secreted protein that binds type 1 IFNs (Symons *et al.*, 1995).
BARF1	EBV	?	Secreted protein that binds CSF-1 (M-CSF) (Strockbine *et al.*, 1998).
GIF	OV	?	Secreted protein that binds GM-CSF and IL-2 (Deane *et al.*, 2000).
MC53, MC54	MCV	?	Secreted protein that binds IL-18 and inhibits IL-18-induced IFN-γ (Smith *et al.*, 2000c).
D7L	VV, EV, CPV, VaV	?	Secreted protein that binds IL-18 and inhibits IL-18-induced IFN-γ (Novick *et al.*, 1999, Smith *et al.*, 2000c).
LMP1	EBV	?	Binds TRAFs. Activation of TNFR p75, B cell proliferation/activation (Mosialos *et al.*, 1995).
C11R (VGF)	VV	?	Epidermal growth factor-like activity. Epithelial cell proliferation (Brown *et al.*, 1995).
MGF	MV	?	Epidermal growth factor-like activity. Epithelial cell proliferation (Brown *et al.*, 1995).
Viral cytokine homologues			
VEGF	OV	VEGF	Stimulates angiogenesis (Savory *et al.*, 2000).
K2	HHV-8	IL-6	Angiogenesis, B cell growth factor (Nicholas *et al.*, 1997, Aoki *et al.*, 1999, Jones *et al.*, 1999).
BCRF-1	EBV	IL-10	Inhibits inflammatory cytokines and IFN-γ (Hsu *et al.*, 1990).
vIL-10	OV, EHV-2	IL-10	Inhibits inflammatory cytokines and IFN-γ (Fleming *et al.*,2000).
cmvIL-10	HCMV	IL-10	Inhibits inflammatory cytokines and IFN-γ (Kotenko *et al.*, 2000).
ORF 13	HVS	IL-17	Stimulates T cell proliferation (Yao *et al.*, 1995).

GRO-α - growth-related oncogene α, IFN - interferon, IL - interleukin, MIP-1α - macrophage inflammatory protein 1α, MDV - Marek's disease virus, MCV - Molluscum Contagiosum, SPV - swinepox virus, CaPV - Capripox virus, MV - myxoma virus, SFV - shope fibroma virus, RPV - rabbitpoxvirus, VV - vaccinia virus, VaV - variola virus, EV – ectromelia virus, CPV - cowpox virus, OV - orf virus, EBV – Epstein-Barr virus, HHV - human herpesvirus, HCMV - human cytomegalovirus, HVS - herpesvirus saimiri, TNF – tumour necrosis factor, TNFR - tumour necrosis factor receptor, VEGF – vascular endothelial growth factor, CSF – colony stimulating factor, GM-CSF, granulocyte macrophage-colony stimulating factor.

1.6.1 Chemokines

Chemokines are soluble, chemoattractant cytokines which function by trafficking leukocytes to sites of tissue damage or viral infection. They are small, disulfide-linked polypeptides, on average about 70 amino acids in length, of which there are over 40 different types. Classification of chemokines are on the basis of conserved structural features, generating four different subfamilies, termed CXC (or α), CC (or β), C (γ), and CX_3C. The CXC chemokines have a single amino acid separating the first two cysteines in a canonical motif, for example IL-8R, in contrast to the CC chemokines which have no amino acid separating the cysteines, for example RANTES. The C subfamily contains only one member, lymphotactin, which has only a single cysteine in the N-terminus of the mature protein. Fractalkine is the sole member of the CX_3C subfamily and is distinguished by containing cysteines separated by three amino acids (Greaves *et al.*, 1997, Bacon *et al.*, 1996, Dairaghi *et al.*, 1998a).

As a large superfamily of proinflammatory proteins, chemokines mediate the activation and migration of a large variety of immune cells by binding to specific G protein-coupled, seven transmembrane receptors (GCRs). The GCR superfamily have several hundred different members specific for a huge array of ligands (Premack *et al.*, 1996). Following receptor interaction, intracellular calcium and activation of other second messenger systems determines the signalling effects of chemokines.

The inflammatory response mediated by chemokines is crucial in the clearance of invading pathogens, therefore, it is expected that in response viruses are likely to move in their own defence mechanisms to maintain their survival.

1.6.2 Chemokine Elements Encoded by Herpesviruses

Anti-chemokine defence strategies employed by viruses involve the production of proteins which appear to regulate chemokines and their receptors. Both the beta and gammaherpesvirus subfamilies encode gene products which contain homology to genes of known chemokine elements (table 1.6a). The alphaherpesviruses do not appear to

have incorporated homologs of chemokine elements into the viral genome.

Murine cytomegalovirus (MCMV) is a betaherpesvirus which expresses MCK1/m131, a viral-encoded chemokine homologue shown to have functional CC chemokine-like activity (Saederup *et al.*, 1999, MacDonald *et al.*, 1997). A MCK1/m131 mutant MCMV exhibited a lower inflammatory response and virus dissemination to target organs in comparison to the wild type virus *in vivo* (Saederup *et al.*, 1999, Fleming *et al.*, 1999). Additionally, MCMV encodes a chemokine receptor, M33, which is conserved and co-linear with the CMV UL33 ORF (Davis-Poynter *et al,* 1997). The M33 protein was shown to be highly expressed during MCMV infection and *in vivo* mice studies have indicated that M33 may be an important factor in trafficking of infected leukocytes or transmission of virus to the salivary glands (Davis-Poynter *et al,* 1997). MCMV also encodes a putative CC chemokine within ORF M131 (MacDonald *et al.,* 1997).

One strain of human cytomegalovirus (HCMV) is predicted to express two CXC chemokine homologues termed vCXC-1 and vCXC-2. The vCXC-1 homolog has been demonstrated to bind the IL-8 receptor CXCR2 and to promote neutrophil migration and degranulation. Furthermore, HCMV has been found to encode four putative chemokine receptors. The GCRs encoded by HCMV are US28, UL33, US27, and UL78. The US28 gene product is very similar to the human CCR1 chemokine receptor (Neote *et al.,* 1993). It has been demonstrated that US28 has the capacity to act as a co-receptor with CD4 for infection by certain subtypes of HIV (Pleskoff *et al.,* 1997). This property of US28 extends the cellular tropism inflicted by HIV infection to other CD4 cells. Thus, HCMV co-infection of HIV positive patients may have an important influence on whether they progress towards AIDS. The GFP gene has been used to disrupt the US28 gene and these studies revealed it is not required for viral replication in cultured cells (Vieira *et al.*, 1998). The US28 GCR has been shown to bind CC chemokines and exhaust the chemokine environment of infected cells *in vitro* (Bodaghi *et al.*, 1998) and has also been demonstrated to mediate smooth muscle cell migration (Streblow *et al.*,

1999). HCMV infection of smooth muscle cells is linked to vascular disease, therefore, the US28 chemokine receptor may function to accelerate the disease by promoting the migration of these cells (Streblow *et al.*, 1999). Immunofluoresence studies on UL33 have demonstrated that UL33 localises to cytoplasmic inclusion structures at late stages of *in vitro* HCMV infection, and that the GCR is expressed on membranes of maturing virions (Marguiles *et al,* 1997). US27 has been observed to exhibit high similarity with US28 and a common notion is that US27 was created by a duplication event with US28. However, the function of US27 is presently unknown, likewise with UL78.

Other betaherpesviruses, such as HHV-6 and HHV-7, encode putative chemokine elements. HHV-6 encodes two potential GCRs, in ORFs U12 and U5 (Isegawa, 1998, Milne *et al.*, 2000, Gompels *et al.,* 1995). The GCR encoded by U12 is related to ORFs UL33, M33, and U12 which are encoded within the genomes of HCMV, MCMV, and HHV-7, respectively. HHV-6 ORF U83 also encodes a putative CC chemokine which has been demonstrated to act as a functional chemokine (Gompels *et al.,* 1995, Zou *et al.*, 1999).

Within the gammaherpesvirus subfamily, several members have been shown to encode potential chemokine elements. Comparative sequence analysis of KSHV identified a CXC chemokine receptor within ORF 74 which is highly related to the human CXC chemokine receptors CXCR1 and CXCR2 (Cesarman *et al.,* 1996). KSHV ORF 74 was also identified using direct hydridisation with an ORF 74/ECRF3 probe (Guo *et al.,* 1997). The IL-8 protein has been demonstrated to bind to the KSHV ORF 74 GCR (Arvanitakis *et al.,* 1997). This GCR stimulates signalling pathways linked to cell proliferation in a constitutive (agonist-independent) manner (Arvanitakis *et al.,* 1997), thus bringing about some confusion over the reason why ORF 74 has ligands which bind at all (eg. IL-8). It is possible that ORF 74 may bind specific chemokines which regulate its signalling activity. A few chemokines including stromal derived factor 1α (SDF-1α) and IFN-γ-inducible protein (IP-10) have been shown to function as inverse agonists by inhibiting the constitutive signalling activity of ORF 74 *in vitro* (Geras-

Raaka *et al.*, 1998). It has recently been demonstrated that KSHV ORF 74 signalling leads to cell transformation and angiogenesis in KSHV-mediated oncogenesis indicating that this HHV-8 GCR is a viral oncogene (Bais *et al.,* 1998). KSHV also encodes three chemokines; vMIP-I, vMIP-II, and vMIP-III. The vMIP-I protein has been demonstrated to contain strong angiogenic properties and is a selective agonist for the CCR8 receptor which is expressed mainly on the TH2 subpopulation of CD4 T cells (Boshoff *et al.,* 1997, Dairaghi *et al.*, 1999, Endres *et al.*, 1999). In contrast, the vMIP-II protein has been characterised as a general inhibitor of a range of chemokines (Kledal *et al.,* 1997, Chen *et al.*, 1998, Sozzani *et al.*, 1998). The vMIP-II protein also has the capacity to inhibit HIV infection with CCR3, CCR5, and CXCR4 receptors as cofactors (Boshoff *et al.*, 1997).

The HVS genome also contains a GCR, ECRF3, which is co-linear with the ORF 74 chemokine receptors of other gammaherpesviruses (Nicholas *et al.,* 1992). The HVS ECRF3 gene has been shown to encode a functional receptor for CC chemokines IL-8, GRO/melanoma stimulating activity (MGSA), and NAP-2 but not for CXC chemokines (Ahuja & Murphy, 1993).

Equine herpesvirus-2 (EHV-2), a member of the gammaherpesvirus subfamily, contains four putative chemokine receptors (Telford *et al,* 1995). Like HVS and HHV-8, comparative sequence analysis revealed that EHV-2 also encodes an ORF 74 homologue which has not been functionally characterised. EHV-2 ORF E1 encodes a GCR which has high similarity with the human CCR1 chemokine receptor (like HCMV) and been shown to be a functional chemokine receptor (Camarda *et al.*, 1999). The third putative chemokine receptor is a duplicate of ORF E1 and the fourth, ORF E6, has an unknown function (Telford *et al.,* 1995).

MHV-68, like many of the gammaherpesviruses mentioned above, also contains a homologue of GCR encoded by ORF 74. If ORF 74 is shown, like HVS and HHV-8 ORF 74, to be a functional IL-8 receptor in transfection experiments, then this would

suggest that the MHV-68 life cycle could be affected by host chemokines signalling through the virally-encoded GCR. Thus, there is a possibility that the high grade lymphomas exhibited by 9% of BALB/c mice infected with MHV-68 (Sunil-Chandra *et al.,* 1994a) may be influenced either in induction or maintenance by host chemokine signalling through the viral GCR. However, it is also feasible that ORF 74 may have a role in the lytic or latent phase of infection. Studies have demonstrated that the MHV-68 ORF 74 has *in vitro* transforming activity which suggests, like HHV-8 ORF 74, the MHV-68 GCR homologue may function as a viral oncogene (Wakeling *et al.*, 2001). A MHV-68 ORF 74 deletion virus for *in vivo* studies would be invaluable for understanding the role of ORF 74 in gammaherpesviruses.

1.6.3 Cytokines

Cytokine is the term given for a large, very diverse family of soluble proteins and peptides which, either under normal or pathological conditions, modulate the functional activities of individual cells and tissues. These proteins also mediate interactions between cells directly and regulate processes taking place in the extracellular environment.

It is very difficult to define a typical cytokine due to the wide diversity of these molecules. Therefore, there are a number of characteristic features cytokines adhere to which allow them to be included in the cytokine family. The majority of cytokines are polypeptides or glycoproteins which are secreted by cells and are usually a relatively small molecular weight. However, many cytokines appear to have a larger molecular weight due to formation of homodimers, homotrimers and heterodimers (e.g. interleukin 12 (IL-12)). Cytokines are rarely constitutively expressed but instead are strictly regulated. Expression normally occurs transiently and is usually in response to an induction signal at the transcriptional or translational level. The biological activities of cytokines are mediated by specific cell surface membrane receptors which can be expressed on virtually all cell types known. Cytokine actions can lead to an increase or decrease in the rate of cell proliferation, change in cell differentiation state and/or a

change in the expression of some differentiated functions. Almost all cytokines are pleiotropic effectors showing multiple biological activities. However, it seems that each cytokine targets at least one of its many activities at haematopoietic cells. More often than not, cytokines show stimulating or inhibitory activities and may also act on other cytokines by synergising or antagonising their actions. Cytokines are not produced by specialised cells which are organised in specialised glands (like hormones). The fact that the same cytokine is often produced by several unrelated cell types and cytokines are secreted suggests that the sites of their expression does not necessarily predict the site at which they exert their biological function. Cytokines are rarely closely related among each other in terms of primary sequences but may share functions.

Within the rapidly growing cytokine family, groups include interleukins, colony stimulating factors, chemokines and interferons. Two different subpopulations of $CD4^+$ T helper cells, termed TH1 and TH2 cells, have been demonstrated in both mouse and humans (Mosmann *et al.,* 1986, Del Prete *et al.,* 1991). TH1 cells produce type 1 cytokines when activated by an antigen or antigen presenting cells (APC), provide limited help for B cell responses and effectively activate cell-mediated responses (Coffman *et al.,* 1988, Hunter *et al.*, 2000). Examples of type 1 cytokines include interleukin-2 (IL-2), interferon-γ (IFN-γ), interleukin-12 (IL-12), and tumour necrosis factor (TNF) (Taniguchi *et al.,* 1993, Waxman *et al.,* 1992, Murray *et al.,* 1985; Nathan *et al.,* 1983; Murray *et al.,* 1983, Dayton *et al.,* 1992, De Maeyer *et al,* 1992, Farrar & Schreiber, 1993, Trinchieri *et al.,* 1993, Van Strijp *et al.,* 1991, Murphy *et al.*, 2000).

TH2 cells produce type 2 cytokines which function to stimulate antibody responses but inhibit cell-mediated or delayed type hypersensitivity (DTH) responses (Hunter *et al.*, 2000). Examples of type 2 cytokines include interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-9 (IL-9), interleukin-10 (IL-10), and interleukin -13 (IL-13) (Howard *et al.,* 1982, Nelms *et al*, 1999, Swain *et al,* 1983, Swain, 1985, Kishimoto, 1989, Renauld *et al.,* 1993, Houssiau *et al,* 1992, Fiorentino *et al.,* 1989, Zurawski & De Vries, 1994, Zurawski *et al.,* 1993, Murphy *et al.*, 2000).

The TH1 and TH2 cytokine pattern strongly contributes to the major functional differences between these two subpopulations of T helper cells. The cytokine pattern assists the cross-regulation of the differentiation and activation of TH1 and TH2 cells during an immune response. The type 1 and type 2 cytokines are only some of the wide variety of cytokines discovered. Many cytokines have an effect on, not only T cells, but also on other cells such as macrophages, B cells and mast cells.

Homologues of the type 2 cytokines, IL-6 and IL-10, have been found in herpesvirus genomes (see section 1.6.4). IL-6 is a multifunctional cytokine which performs a plethora of biological activities. This cytokine regulates immune responses, haematopoiesis and acute phase reactions, thus playing a central role in host defense mechanisms (Kishimoto, 1989; Van Snick, 1990; Akira *et al.,* 1993). IL-6 was first discovered as a T cell-derived lymphokine that induces final maturation of B cells into antibody producing cells (Muraguchi *et al,* 1981; Hirano *et al.,* 1985). In addition, IL-6 also acts as an essential factor in activation for resting T cells. In the presence of IL-2, IL-6 induces the differentiation of mature and immature T cells into cytotoxic T cells (CTL) (Takai *et al.,* 1988, Okada *et al.,* 1988). IL-10 has a wide variety of functions in the immune system. This secreted protein was first discovered when it was observed that the supernatant from activated T cells had an inhibitory effect on the secretion of cytokines in TH1 cells which included IFN-γ, IL-2 and TNF-β (Fiorentino *et al.,* 1989, D'Andrea *et al,* 1993, Gerard *et al.,* 1993, Taga *et al.* 1992). The inhibitory effect on IFN-γ production by IL-10 is indirect and appears to be the result of a suppression of IL-12 synthesis by accessory cells (D'Andrea *et al.,* 1993). However, reduction of IL-12 synthesis is not the only mechanism whereby IL-10 reduces cytokine synthesis in TH1 cells. It is possible that IL-10 downregulates other co-stimulatory molecules. IL-10 also has biological effects on a variety of other cell types including macrophages, NK cells, B cells and mast cells (Mosmann *et al.,* 1994). Recently, IL-10 has been shown to inhibit the down-regulation of inflammatory chemokine receptors in dendritic cells and monocytes exposed to an inflammatory microenvironment. This has generated

functional decoy receptors which are able to mop up chemokines and act in an anti-inflammatory manner (D'Amico *et al.*, 2000).

1.6.4 Cytokine Elements Encoded by Herpesviruses

It is not a surprise that, in addition to chemokine elements, the beta and gamma herpesviruses have been demonstrated to encode potential cytokine elements (table 1.6b). Homologues of cytokine elements are employed to circumvent detection and combat the host immune defence mechanisms.

Within the EBV genome, human IL-10 exhibits 78% amino acid identity overall to the protein encoded by the BCRF1 gene. The BCRF1 gene product has a molecular weight of 17 kD and is produced and secreted during the lytic phase of EBV infection (Hsu *et al.*, 1990, Stewart & Rooney, 1992; Swaminathan *et al.,* 1993; Ryon *et al.,* 1993; Stewart *et al.,* 1994). The viral IL-10 shares similar but not identical biological activities with human IL-10. They both inhibit monocyte/macrophage activities, thereby inhibiting synthesis of cytokines from these cells. Human IL-10 and BCRF1 also downregulate IFN-γ synthesis by T cells and NK cells which disrupts IFN-γ-induced upregulation of MHC class II molecules on APC. However, a positive effect is that they both stimulate proliferation and antibody secretion from human B cells activated through binding of their CD40 surface antigen (Rousset *et al.,* 1992). Thus, BCRF1 most likely functions to assist EBV in escaping host surveillance by interfering in the antiviral effects of IFN-γ while facilitating EBV replication through the activation of resting B cells (Miyazaki *et al.,* 1993).

A viral IL-10 homolog was recently discovered to be encoded by the HCMV ORF UL111a and termed cmvIL-10 (Kotenko *et al.*, 2000). Although the cmvIL-10 only exhibits 27% amino acid homology to its human counterpart, it has been shown to bind the human IL-10 receptor and compete for human IL-10 binding sites (Kotenko *et al.*, 2000).

HVS has been shown to encode a number of cytokine elements. Previous work had isolated a cytokine-like protein termed CTLA-8 from activated T cells, which encoded a 15 kD secreted protein. Comparison of the deduced amino acid sequence of CTLA-8 with others in a sequence database revealed significant homology between CTLA-8 and HVS ORF 13. CTLA-8, now referred to as interleukin-17 (IL-17), exhibits 57% amino acid identity overall with its viral homologue (Spriggs, 1994). It has been demonstrated that the viral IL-17 homologue exhibits pleiotropic cytokine-like activity including the induction of NFκB and co-stimulation of T-cell proliferation (Yao *et al.,* 1995; Fossiez *et al.,* 1996). The function of the viral homologue has not been elucidated but it is likely this protein will have a role in assisting HVS infectivity of T lymphocytes.

KSHV encodes a viral IL-6 homologue which has been shown to stimulate the growth of KSHV-infected primary infusion lymphoma (PEL) cells, promote hematopoiesis, and act as an angiogenic factor through the induction of vascular endothelial growth factor (VEGF) (Nicholas *et al.*, 1997, Aoki *et al.*, 1999, Osborne *et al.*, 1999, Jones *et al.*, 1999).

No genes with homology to known cytokine elements have been found in MHV-68. However, given the propensity of the gammaherpesviruses to abduct cytokines, it is likely that proteins with cytokine functions and properties will be encoded within the genome. Candidates are the M genes which have no known homologues within the sequence databases. Analysis of the M genes indicate that the M4 ORF has properties which are consistent with it functioning as a cytokine.

1.7 Aim

The aim of my project is to functionally characterise the M4 gene and elucidate its role in the pathogenesis of MHV-68.

Protein expression would allow the generation of rabbit anti-sera which would be an invaluable tool in both *in vitro* and *in vivo* immunostaining studies to determine the

expression of M4. The addition of M4 protein directly on cells such as epithelial and B cells would allow us to determine if M4 has any proliferative effects which would be of assistance in functional studies. RNA expression studies would provide an indication of the time post-infection of when the M4 gene is transcribed and whether it can be termed an immediate early, early, late or a latency gene.

The generation of a M4 recombinant virus and use of this for infections *in vivo* and *in vitro* studies would be extremely beneficial for resolving the function of this gene.

Chapter Two:

Materials & Methods

2.1 DNA Studies

2.1.1 DNA Sequencing

All DNA sequencing was kindly carried out by Mr. Ian Bennet using a LI-COR DNA Sequencer 4000 (MWG-Biotech).

2.1.2 Restriction Enzyme Digestion

All restriction enzymes used were purchased from Gibco BRL and used according to the manufacturer's instructions in the supplied buffers. The standard reaction mix consisted of 1 unit of a restriction enzyme to 1μg of target DNA in the appropriate buffer (total volume ranging from 20-100μl). All solutions were dispensed into a 1.5ml microcentrifuge tube and incubated at 37 °C for 2-3 hours.

2.1.3 Dephosphorylation of Linearised DNA

The 5' phosphate of the cut ends of plasmid DNA were dephosphorylated by the addition of 10 units of calf intestinal alkaline phosphatase (Boehringer) to the restriction digest reaction mix. This reaction mix was incubated for a further 20min at 37 °C and stopped by phenol/chloroform extraction as described in 2.1.5.

2.1.4 Ligation of DNA Fragments

Ligation of DNA fragments was performed using T4 DNA ligase (Gibco BRL). Purified DNA fragments were mixed with the cut DNA vector at ratios in the range of 3:1 – 6:1 respectively in ligase buffer (50mM Tris-HCL [pH 7.6], 10mM $MgCl_2$, 1mM dithiothreitol, 5% (w/v) polyethylene glycol-8000 (Gibco BRL)) and 1unit of T4 DNA ligase at a final volume of 20μl. This reaction mix was incubated at 4 °C overnight.

2.1.5 Phenol Chloroform Extraction and Ethanol Precipitation

To clean DNA solutions, an equal volume of phenol/chloroform (1:1) solution (Amresco, USA) was added, vortexed and then centrifuged for 5min at 13,000*g*. The upper phase containing the DNA was removed and aliquoted into a fresh tube containing an equal volume of chloroform. The tube was again vortexed and centrifuged for 5min

at 10,000*g*. The upper phase was removed and aliquoted into a fresh tube containing 1/10 volume of 3M sodium acetate and then 3 volumes of ethanol were added. The tube was incubated at -20°C for 1 hour to allow the DNA to precipitate. The tube was centrifuged for 10 min at 10,000*g* to pellet the DNA. The supernatant was removed and 500µl of 70% ethanol was used to wash the pellet. The tube was centrifuged for 3 min at 10,000*g*, ethanol was removed and the pellet was resuspended in 50µl of Tris EDTA (TE) buffer.

2.1.6 Agarose Gel Electrophoresis

To form a 0.7% TAE gel, 100ml of 1X TAE buffer (40mM Tris-acetate [pH7.8] and 1mM EDTA) and 0.7g of SeaKem LE agarose (Flowgen) was added to a microwavable bottle. Contents were boiled in a microwave for several minutes until the agarose had dissolved. The solution was left to cool for 20min and then 3µl of 10mg/ml ethidium bromide (Sigma) was added to the bottle and swirled. The gel solution was poured into a Minnie the Gel Cicle submarine agarose gel unit HE33 (Hoefer) or a Biorad mini gel kit and left to set. 2µl of 10X loading buffer (15% w/v ficoll type 400, 0.25% bromophenol blue, 0.25% xylene cyanol or 0.25% orange G in H_2O) was added to the DNA samples and loaded into the wells of the set TAE gel. 1µl of a 1Kb DNA ladder (Gibco BRL) was added to 15µl of loading buffer and this was added to a well for sizing the DNA fragments. The gel tank was filled with 1X TAE buffer and the gel was electrophoresed for 1 hour at 70 volts using a Bromma power pack supply (LKB). DNA bands were visualised using a UV transilluminator.

2.1.7 Extracting DNA from Agarose Gels

DNA separated on agarose gels was extracted by placing the gel slice containing the DNA into a large well of a 1% TAE gel and covered with PEG solution (20% (w/v) PEG in 1X TAE, 200ng of ethidium bromide (Sigma)). The 1% TAE gel was placed in a Biorad Mini-sub cell and electrophoresed at 100V for 2 minutes, during which the DNA moved from the gel slice into the surrounding PEG solution. The PEG solution was

removed and extracted with phenol chloroform. The DNA was purified and concentrated from the aqueous phase using a Microcon column (Amicon).

2.1.8 Quantitation of DNA

DNA concentration was quantified using a CECIL spectrophotometer. The buffer in which the nucleic acid was eluted in was used to zero the machine. The nucleic acid was diluted from 1/50 – 1/500 and measured at OD_{260}.

Nucleic acid concentration was calculated using the following formula:

DNA concentration (μg/ml) = OD_{260} x dilution x 50

2.1.9 Colony transformation

BL21(DE3) bacteria (Novagen) were streaked onto LB agar plates (1% bacto tryptone, 0.5% bacto yeast extract, 1% NaCl), 1.5% bacteriological agar (Oxoid Ltd) containing 10-20mM Mg^{2+}. The plates were incubated overnight at 37°C to establish colonies. A tungsten inoculating loop was used to pick 4 colonies and these were dispersed in 200μl of TFB (100mM KCL, 45mM $MnCl.4H_2O$, 10mM $CaCl_2.2H_2O$, 3mM $HACoCl_3$, 10 mM K-MES (2[N-morpholino]ethone sulphonic acid, [pH6.3]) by vigorously vortexing with a rotamixer (Hook and Tucker Ltd) in a 15ml polybutadeine styrene conical tube (Nunc, USA). The tube was incubated on ice for 30 min. 7μl of DnD solution (1M dithiothreitol dimethyl sulfoxide, 90% DMSO, 10mM potassium acetate) was added into the centre of the solution and the tube was swirled for a few seconds and placed on ice for 15 min. An additional 7μl of DnD was added, the tube was again swirled gently, and incubated on ice for 20 min. 1μl of pET-22b(+) vector (Novagen) containing the M4 gene (provided by James P. Stewart) was added to the solution and incubated on ice for 10-40 min. The cells were heat shocked by placing the tube in a 42°C water bath for 90 sec and then chilled on ice for 2min. 800μl of SOC medium (2% bacto tryptone, 0.5% bacto yeast extract, 10mM NaCl, 2.5mM KCL, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose) was added to the cells and then they were incubated in the Orbital Shaker (Forma Scientific Inc., USA) at 37°C for 60 min. The cells were gently centrifuged and

resuspended in 250µl of SOC medium. The bacteria were plated out on agar plates composed of LB, bacteriological agar, 10-20mM Mg^{2+}, and 50µg/ml of carbenecillin (Sigma).

2.1.10 Transformation of Epicurian Coli Ultracompetent Cells

Epicurian Coli ultracompetent cells (Stratagene) were transformed according to manufacturer's instructions as follows. Cells were thawed on ice and gently mixed by hand. 50µl of the cells were aliquoted per transformation into each pre-chilled 15ml polybutadeine styrene conical tube. β-mercaptoethanol was added to 25mM concentration to each tube. The contents of the tubes were swirled gently and incubated on ice for 10 min, swirling every 2min. 2µl of DNA ligation mix was added to each tube and swirled gently and the tubes were left to incubate on ice for 30min. The tubes were heat-pulsed in a 42°C water bath for exactly 30sec and left on ice for 2min. 0.9ml of preheated (42°C) NZY^+ broth was added and incubated in the tubes at 37°C for one hour in the Orbital Shaker. Before plating the transformation mixture, the cells were concentrated by gentle centrifugation for 10min and resuspended in 200µl of NZY^+ broth. The plates were incubated at 37°C overnight.

2.1.11 Small Scale Purification of Plasmid DNA

Colonies were grown on an agar plate composed of LB, bacteriological agar and 50µg/ml of carbenecillin. A colony was picked containing pET-22b(+) vector with the M4 gene insert. The colony was inoculated into a universal tube (Bibby Sterilin Ltd, UK) containing 6ml of LB and 50µg/ml of carbenecillin. The culture was grown overnight in the Orbital Shaker at 37°C and then centrifuged for 10min at 500*g*. The pellet was resuspended in 200µl of a solution containing 1M glucose, 1M Tris pH8.0, 0.5M EDTA, [pH8.0]. The solution was transferred to a 1.5ml microcentrifuge tube and 400µl of a solution containing 0.2M NaOH and 1% SDS was added. The tube was left at room temperature for 10min. 300µl of a solution containing 2.7M potassium acetate, [pH5.5], was added and the tube was left on ice for 10min. The tube was centrifuged for

3min in a microcentrifuge (MSE) and 800µl of the supernatant was removed and aliquoted into a fresh tube. 2.5µl of a 20µg/ml stock of RNAse A was added and the tube was left to incubate at 37°C for 1 hour. DNA was phenol chloroform extracted and ethanol precipitated as described in section 2.1.5.

2.1.12 Polymerase Chain Reaction (PCR)

PCR reactions were performed using either *Taq* DNA plymerase (Gibco BRL) or *PfuTurbo* DNA polymerase (Stratagene).

A PCR reaction using *Taq* DNA polymerse was performed in a final volume of 100µl containing 1µl of primer mixture stock (10µM each), 1µl of 10mM dNTP mixture (10µl of each dNTP added to 60µl of diH_2O), 3µl of 50mM $MgCl_2$, 10µl of 10X PCR buffer, 1µl target DNA (amounts ranged from 1-100ng) and 1µl of *Taq* DNA polymerase.

A PCR reaction using *PfuTurbo* DNA polymerase was performed in a final volume of 50µl containing 1µl of primer mixture stock (10 µM each), 1µl of 10mM dNTP mixture (10µl of each dNTP added to 60µl of diH_2O), 1µl target DNA (amounts ranged from 1-100ng), 1µl of *PfuTurbo* DNA polymerase and 10µl of 10X cloned PPU buffer.

An Omnigene PCR machine (Hybaid) was used for the temperature cycles. The standard PCR cycling program involved an initial stage of denaturation for 3min at 94°C without *Taq* DNA polymerase. The hotstart method was used which involved aliquoting 1µl of *Taq* DNA polymerase to the tubes while holding the temperature of the PCR mixture at 80°C. 30 cycles were performed of denature for 45s at 94°C, anneal for 45s at 55°C and extend for 2min at 72°C. After the 30 cycles, the tube was incubated for an additional 5min at 72°C to complete the extension. To allow the Omnigene PCR machine to cool down, the tube was incubated for 30min at 35°C. The PCR products were analysed by agarose gel electrophoresis as described in section 2.1.6.

2.1.13 Calculation of Primer Annealing Temperature

For PCR using *Taq* DNA polymerase, the annealing temperature for primers was calculated using the following formula:

annealing temperature (^{o}C) = $4(B_G+B_C) + 2(B_A+B_T) - 5$

For PCR using *PfuTurbo* DNA polymerase, the annealing temperature for primers was calculated using the following formula:

annealing temperature (^{o}C) = $4(B_G+B_C) + 2(B_A+B_T)$

B equals the number of guanosine (G), cytosine (C), adenine (A) and thymidine (T) bases in the primer complementary to the target DNA in the first round of PCR.

2.1.14 Calculation of PCR Extension Time

When using *Taq* DNA polymerase and *PfuTurbo* DNA polymerase, extension times were calculated on the basis of 1 minute/kb.

2.1.15 Primers

Oligonucleotides were purchased from MWG biotech. Primers which have bases complementary to the target sequence during the first cycle of PCR are in italics.

M4A – Within M4 gene (sense)

5' – GCGCGGATCCGACAC*CTGGAGAAGATGATGATATTC* – 3'
*Bam*HI
5' location on MHV-68 genome: 8617

M4B – Within M4 gene (antisense)

5' – CGCGGAATTCGG*TTCTAGAAAGTCATAAATCTCAATACC* – 3'
*Eco*RI
5' location on MHV-68 genome: 9786

M4A & M4B product size: 1169bp
Target: MHV-68 genome
PCR conditions with *Taq* polymerase: 55^{o}C annealing temperature, 2min extension time, 30 cycles.

M4Arev - Within M4 gene (antisense)

5' – CGCGAATTC*GGCAGTCGCATAACCATGTCCACG* – 3'
*Eco*RI
5' location on MHV-68 genome: 9176

M4A & M4Arev product size: 559bp
Target: MHV-68 genome
PCR conditions with *Taq* polymerase: 55°C annealing temperature, 2min extension time, 30 cycles.

M4Bfor – Within M4 gene (sense)

5' – CGCGGATCCG*GTGGACATGGTTATGCGACTGA*– 3'
*Bam*HI
5' location on MHV-68 genome: 9156

M4B & M4Bfor product size: 630bp
Target: MHV-68 genome
PCR conditions with *Taq* polymerase: 55°C annealing temperature, 2min extension time, 30 cycles.

M4pc - Within M4 gene (sense)

5' – GCGCAAGCTT*GGTCCGGCCTCACATCAT* - 3'
*Hin*dIII
5' location on MHV-68 genome: 8503

M4B & M4pc product size: 1284bp
Target: MHV-68 genome
PCR conditions with *pfu* polymerase: 40°C annealing temperature, 4min extension time, 20 cycles.

M41F - Left of M4 gene (sense)

5' – CGCGCTCGAG*TGTGGTTACAGTCTGCCACC* - 3'
*Xho*I
5' location on MHV-68 genome: 7833

M41R - Left of M4 gene (antisense)

5' – AAGCTTGTCGACGAATTC*CACTCATCTGACTTGCTGCATAC* – 3'
*Hin*dIII – *Acc*I ----*Eco*RI
5' location on MHV-68 genome: 8408

M41F & M41R product size: 575bp

Target: MHV-68 genome
PCR conditions with *Taq* polymerase: 57°C annealing temperature, 2min extension time, 30 cycles.

M42F - Right of M4 gene (sense)

5' – GAATTCGTCGACAAGCTT*CTAGAATAACTGTACCCTGTTAAG* – 3'
*Eco*RI ----*Acc*I----*Hin*dIII
5' location on MHV-68 genome: 9780

M42R(2) - Right of M4 gene (antisense)

5' – GCGCGGATCC*TGGTCACCTCTCGGGTGTTA* - 3'
*Bam*HI
5' location on MHV-68 genome: 10401

M42F & M42R(2) product size: 621bp
Target: MHV-68 genome
PCR conditions with *Taq* polymerase: 57°C annealing temperature, 2min extension time, 30 cycles.

M41F & M42R(2) product size: 1172bp
Target: M41F & M41R product and M42F & M42R(2) product
PCR conditions with *Taq* polymerase: 57°C annealing temperature, 2min extension time, 30 cycles.

M4LH1 – Right of M4 gene (sense)

5' – *GTACTATATGGCACCAGC* – 3'

5' location on MHV-68 genome: 7786

GFP1 – Within GFP gene (sense)

5' – *GCGCGAATTCGTTCATAGCCCATATATGGAG* – 3'

GFP2 – Within GFP gene (antisense)

5' – *GCGCGAATTCCGCGTTAAGATACATTGATGAG* – 3'

M4LH1 & GFP2 product size: 1600bp
Target: M4KO DNA
PCR conditions with *Taq* polymerase: 55°C annealing temperature, 2min extension time, 30 cycles.

GFP1 & GFP2 product size: 1400bp
Target: M4KO DNA
PCR conditions with *Taq* polymerase: 55°C annealing temperature, 2min extension time, 30 cycles.

MHV76A – Right of M4 gene (sense)

5' – GCGCGGATCC*CATGTGCTACCTCTGTG* – 3'
*Bam*HI
5' location on MHV-68 genome: 7414

MHV76B – Left of M4 gene (antisense)

5' – GCGCGAATTC*GTACCGTCTGAGTGACTG* – 3'
*Eco*R1
5' location on MHV-68 genome: 10914

MHV76A & MHV76B product size: 3500bp
Target: MHV-68 genome
PCR conditions with *Taq* polymerase: 51°C annealing temperature, 2min extension time, 30 cycles.

M4GFP76 – Right of M4 gene (antisense)

5' – GCGC*GACGCGACTAGCATGAGGA* – 3'

5' location on MHV-68 genome: 11191

M4A & M4GFP76 product size: 2574bp
Target: M4 knock in DNA
PCR conditions with *Taq* polymerase: 55°C annealing temperature, 2min extension time, 30 cycles.

CMV1 – within pEGFP-C1 plasmid (sense)

5' – CGCGGGATCC*GTTCATAGCCCATATATGGA* - 3'
***Acc*I**
5' location on pEGFP-C1 plasmid (Clontech): 35

SV40 – within pEGFP-C1 plasmid (antisense)
5' – GCGCAGATCT*CGCGTTAAGATACATTGATGAG* – 3'
*Acc*I
5' location on pEGFP-C1 plasmid (Clontech): 1646

CMV1 & SV40 product size: 1631bp

Target: pEGFP-C1 plasmid (Clontech)
PCR conditions with *Taq* polymerase: 55°C annealing temperature, 3min extension time, 35 cycles.

CMVBam – within pEGFP-C1 plasmid (sense)

5' – CGCGGGATCC*GTTCATAGCCCATATATGGA* - 3'
*Bam*HI
5' location on pEGFP-C1 plasmid (Clontech): 35

SV40Bgl – within pEGFP-C1 plasmid (antisense)
5' – GCGCAGATCT*CGCGTTAAGATACATTGATGAG* – 3'
*Bgl*II
5' location on pEGFP-C1 plasmid (Clontech): 1646

CMVBam & SV40Bgl product size: 1631bp
Target: pEGFP-C1 plasmid (Clontech)
PCR conditions with *Taq* polymerase: 55°C annealing temperature, 3min extension time, 35 cycles.

MA1 – Within gp150 gene (sense)

5' – GGACCATGGGA*TTTCTGGGGAATCACAACTTAG* – 3'
*Nco*I
5' location on MHV-68 genome: 69,520

MA2 – Within gp150 gene (antisense)

5' – GCTCTCG*AGGGTAGAAATGTCTGGAACGG* – 3'
*Xho*I
5' location on MHV-68 genome: 70, 826

MA1 & MA2 product size: 1324bp
Target: MHV-68 genome
PCR conditions with *Taq* polymerase: 50°C annealing temperature, 2min extension time, 25 cycles.

neoF – Within neomycin resistance gene (sense)

5' – *GTGCTCGACGTTGTCACTGAA*– 3'

neoR – Within neomycin resistance gene (antisense)

5' – *CTGATGCTCTTCGTCAGATC* – 3'

neoF & neoR product size: 257bp
Target: pcDNA3.1/V5/HIS/TOPO plasmid (Invitrogen)
PCR conditions with *Taq* polymerase: 55°C annealing temperature, 2min extension time, 30 cycles.

Murine β-actin 1 – Within β-actin gene (sense)

5' – *TGTGATGGTGGGGAATGGGTCA*– 3'

Murine β-actin 2 – Within β-actin gene (antisense)

5' – *TTTGATGTCACGCACGATTTC*– 3'

Murine β-actin 1 & Murine β-actin 2 product size: 514bp
Target: M4KI cDNA
PCR conditions with *Taq* polymerase: 55°C annealing temperature, 2min extension time, 30 cycles.

2.1.16 PCR Product Purification

The Wizard PCR Preps DNA Purification System (Promega, USA) was used to purify the PCR product. 100µl of direct purification buffer was added to a 1.5ml microcentrifuge tube containing 30-300µl of the PCR reaction mix. 1 ml of resin was added to the tube and this was vortexed 3 times over 1 minute. For each PCR product, one Wizard minicolumn was prepared. The plunger from a 5 ml disposable syringe was removed and set aside and a minicolumn was placed at the end of the syringe. The resin/DNA mix was pippeted into the syringe barrel and the syringe plunger was inserted slowly and gently to push the slurry into the minicolumn. The syringe was detached from the minicolumn and the plunger was removed from the syringe. The syringe barrel was reattached to the minicolumn and 2ml of 80% isopropanol was added to the syringe barrel. To wash the minicolumn the syringe plunger was again inserted into the syringe barrel and pushed the isopropanol through slowly. The syringe was removed and the minicolumn was transferred to a 1.5ml microcentrifuge tube. The minicolumn was centrifuged at 10,000*g* to dry the resin. The minicolumn was transferred to a new microcentrifuge tube, 50µl of TE buffer was applied and left for 1min. Centrifugation of the minicolumn for 20sec at 10,000*g* was performed to elute the bound DNA fragment which was stored frozen.

2.1.17 M4 KO Recombinant Construction

The following PCRs were performed using the standard PCR protocol. A 575bp flanking sequence to the left of M4, termed M41 was generated by PCR, using primers M41F and M41R. The M41F and M41R primers contained a *Xho*I and a *Hind*III-*Acc*I-*Eco*RI restriction enzyme site, respectively, at their 5' ends. The same was performed for a 621bp right flanking sequence termed M42, using primers M42F and M42R(2). The M42F and M42R(2) primers contained a *Eco*RI-*Acc*I-*Hind*III and *Bam*HI restriction enzyme site, respectively, at their 5' ends. Both M41 and M42 PCR products were analysed on a 0.8% (w/v) agarose gel.

A DNA fragment containing the left (M42) and right (M41) flanking sequence of M4 was generated by PCR via the *Hind*III-*Acc*I-*Eco*RI site. 1µl of the M41 and M42 PCR reactions was used as template DNA in a PCR with the primers M41F and M42R(2) to generate a DNA fragment of 1172bp which was detected on a 0.8% agarose gel. The M41/M42 DNA was purified using a PCR wizard clean kit (Promega). The entire PCR product was cut with *Xho*I and *Bam*HI restriction enzymes for 3 hours at 37°C and then purified again. All enzymes used were purchased from GibcoBRL. pKS vector was cut with the same restriction enzymes and extracted with phenol chloroform using the standard protocol.

All cloning was performed using standard cloning protocols unless otherwise stated. The M41/M42 DNA was ligated into the pKS vector cut with the appropriate enzymes. The ligated vector was transformed into XL1-blue competent cells (Stratagene) using the manufacturer's protocol and the resultant colonies were picked and grown overnight in LB broth. DNA was prepared from mini cultures (5-10ml) using a miniprep kit (Qiagen) and checked for the correct clone using restriction analysis.

The pKS vector containing the M41/M42 DNA insert was digested with *Acc*I for 4 hours at 37°C. 1µl of alkaline phosphatase (Boehringer Mannheim) was added to the digest

for the final 20 mins of incubation and extracted with phenol chloroform. The green fluorescent protein (GFP) gene was obtained by *Acc*I digestion of pAC8 vector (kindly provided by Dr. Douglas J. Roy). pAC8 was derived from pEGFP-C1 (Clontech) by deletion of the multiple cloning site. The pAC8/GFP vector was digested with AccI for 3 hours at 37°C. The entire digest was separated on a 0.8% agarose gel and the band containing the GFP gene was isolated and the DNA was extracted as described in section 2.1.7.

The GFP DNA was cloned into the corresponding sites in the pKS/M41/M42 and DNA was prepared using a endotoxin-free maxi-prep kit (Qiagen) according to the manufacturer's protocol. DNA was quantitated by UV spectroscopy (CECIL). The M4 knock out cassette was cut out from the pKS vector with *Xho*I and *Bam*HI. This crude digest was used for transfection.

2.1.18 M4 KI Recombinant Construction

A pUC13 plasmid containing a 1.2kb *Pst*I fragment from the MHV-68 terminal repeat (kindly provided by Dr. James P. Stewart) was sequenced to determine the orientation of the terminal repeat (TR). For our purposes we required the TR to read from left to right. However, the sequence revealed the TR was reading in the opposite direction. The TR was cut with *Pst*I and re-ligated into the pUC13 plasmid. All clones revealed the TR was ligating from right to left indicating preferential orientation with respect to the *lacZ* promoter. To overcome this problem the PstI fragment was cloned into pUC18 which contains the multiple cloning site in the opposite orientation to the *lacZ*. Orientation was confirmed by restriction analysis.

The following PCRs were performed using the standard PCR protocol. DNA containing the M4 gene, a 1124bp flanking sequence to the left of M4 and a 1129bp flanking sequence to the right of M4, were generated by PCR using primers MHV76A and MHV76B. The MHV76A and MHV76B primers contained 5' *Bam*HI and *Eco*RI

restriction enzyme sites, respectively. M4 was digested with *Bam*HI and *Eco*RI and cloned into its corresponding site in the pUC18 vector.

The pEGFP-C1 (Clontech) plasmid with the multiple cloning site deleted (kindly provided by Dr. James P. Stewart) was used as a template for PCR using primers SV40Bam at the 5' end and CMVBglII at the 3'end (see appendix). The 1.6kb GFP fragment was digested with *Bam*HI and *Bgl*II and ligated into its corresponding sites in the pUC18 plasmid. There are two possible orientations which the GFP fragment can enter. The desired orientation is for the 3' end of GFP containing the *Bgl*II site to ligate into the BamHI site. The ligations were transformed into *E.coli* Solopack-Gold cells (Stratagene) using the manufacturer's standard protocol. Resultant colonies were grown using the standard protocol and screened using restriction analysis.

DNA from the correct orientation was prepared using a endotoxin-free maxi-prep kit (Qiagen) according to the manufacturer's protocol. DNA was quantitated by UV spectroscopy (CECIL). The M4/GFP/TR cassette was excised from the pUC18 vector with *Eco*RI and *Hind*III. This crude digest was used for transfection.

2.1.19 Extraction of Viral DNA

T175 flasks containing cells and plaques were washed x 2 in PBS and 10ml of extraction buffer (0.1M EDTA, 0.5% SDS, 0.2M Tris-HCL [pH8.0]) dispensed into the flasks. Cells were scraped and transferred to 15ml polypropylene conical tubes. The procedure was continued using pipette tips with ends cut off to prevent shearing of DNA. 20μg/ml of RnaseA (Sigma) were added to the tubes and incubated for 1hr at 37°C. 100μg/ml of proteinase K (Roche) were added to the tubes and incubated at 56°C overnight. The DNA was extracted using phenol/chloroform x3 and chloroform x1. After every addition of phenol or chloroform, the tubes were placed on a rotating mixer for 15min and centrifuged at 3.5K for 15 min at 4 °C. The DNA was precipitated in 2 volumes of 100% ethanol and 0.1 volume of 3M sodium acetate at room temperature. The DNA were centrifuged as before and the pellets were washed with 70% ethanol x2. Excess

ethanol was removed and the pellet left to dry for 5min. The DNA was resuspended in 200µl of distilled H_20 and quantitated.

2.1.20 Radioactive Labelling of DNA Fragments

DNA radiolabelled with the isoptope ^{32}P was used to probe Southern and northern blots. The DNA was labelled using a Random Primed DNA Labelling Kit (Boehringer Mannheim). 25 ng of DNA in 9 µl of distilled water was incubated for 10min at 95°C and cooled on ice. The DNA was added to a 1.5ml microcentrifuge tube containing 1µl of dATP, 1µl of dGTP, 1µl of dTTP, 2µl of reaction mixture, 5µl of [^{32}P]-dCTP, and 1µl of Klenow enyme (2 units/µl). The tube was incubated for 40min at 37°C. The labelled DNA was increased to a volume of 100µl with TE and purified on a nick column (Amersham Pharmacia). The column was equilibrated with 3ml of TE and the 100µl of labelled DNA was added to the column. 400µl of TE was added and the flow-through was discarded. Another 400µl of TE was added and the flow-through collected contained the labelled DNA probe. The probe was denatured with 0.1 volume of 1M NaOH for 5min at 37°C.

2.1.21 Southern Analysis

5µg of each digested DNA sample and a 1kb DNA ladder were loaded and electrophoresed in a 0.7% agarose gel as described in section 2.1.6 for 3-4hr at 70-80V.

The gel was washed with distilled water, soaked in a 0.5M NaOH, 1M NaCl solution for 20min x 2, soaked in 0.5M Tris-HCL pH7.5, 1.5M NaCL solution for 20min x 2, and washed again in distilled water.

2.1.22 Transfer of Nucleic Acids by Blotting

A large Biorad gel kit was used as the blotting apparatus and was filled halfway with 20X SSC. Two long strips of 3 MM chromatography paper cut to the width of the gel were soaked with 20X SSC and placed on the gel kit so that the ends were submerged

into the 20X SSC sitting at the bottom of the kit. The gel was placed back on the paper with the face downwards. Magnagraph nylon transfer membrane (MSI) cut to the size of the gel was soaked in 20X SSC and placed on top of the gel. This was followed by two sheets of 3MM chromatography paper cut to the size of the gel and soaked in 20X SSC, a stack of blotting paper and a weight. Clingfilm was placed around the edges of the gel to prevent the paper under and above the gel from touching resulting in the blot short-circuiting. The nucleic acids are drawn up onto the membrane by capillary overnight.

The nucleic acid on the membrane was immobilised by placing the membrane in a Stratalinker and exposing to a source of ultraviolet irradiation. The membrane was wrapped in paper and clingfilm and stored at -80°C.

2.1.23 Southern Blots Probed with DNA

Prior to probing, the nylon membrane was pre-hybridised with a solution containing 6X SSC and 5% milk (Marvel). The membrane and pre-hybridisation solution was incubated in a sealed plastic bag or in a hybridisation bottle for 4 hr at 68°C.

To hybridise the membrane with the DNA probe, the pre-hybridisation solution was replaced with a hybridisation solution containing 6X SSC, 5% milk and the labelled DNA probe. The membrane, hybridisation solution and labelled probe were incubated overnight at 68°C.

The membrane was subjected to a series of washes to remove non-specific binding at a high stringency. The first washes were 2 x 15min at room temperature with a solution containing 5X SSC, 0.5% SDS. The second washes were 2 x 15min at 37°C with a solution containing 1X SSC, 0.5% SDS. The final washes were 3 x 15min at 65°C with a solution containing 0.1 SSC, 1% SDS.

To detect labelled probe, the membrane was covered in clingfilm and placed in a cassette containing a screen and Kodak Xomat film (Sigma). The cassette was stored overnight at -80°C. The film was developed using a x-ray developer.

2.2 RNA Studies

2.2.1 Total RNA extraction

The RNAzol B method (Sigma) was used to extract total RNA. 0.2ml of chloroform was added per 2ml of homogenate in the universal tubes. The tubes were shaken vigorously but not vortexed and incubated on ice for 5min. The suspensions were transferred to 1.5ml microcentrifuge tubes and centrifuged for 15min at 12,000*g* at 4°C. The top aqueous phase of the tubes were transferred to fresh tubes, an equal volume of isopropanol was aliquoted into the tubes and the samples were stored for 15min at 4°C. The tubes were centrifuged for 15min at 12,000*g* at 4°C and the supernatants were removed. The RNA pellets were washed once with 75% ethanol by vortexing and subsequent centrifugation for 8min at 7500*g* at 4°C. All traces of ethanol were removed and the pellets were resuspended in 100µl of RNase-free H_2O. Tubes with the same RNA timepoints were pooled and stored at -80°C.

2.2.2 Quantitation of RNA

RNA concentration was quantified using a CECIL spectrophotometer. The buffer in which the nucleic acid was eluted in was used to zero the machine. The nucleic acid was diluted from 1/50 – 1/500 and measured at OD_{260}.

Nucleic acid concentration was calculated using the following formula:

RNA concentration (µg/ml) = OD_{260} x dilution x 40

2.2.3 Removal of DNA from RNA Preparations

The Ambion DNase free kit was used. RNA samples were diluted to 2µg/µl. A 0.1 volume of 10X DNase I buffer and 4 units of DNase I was added to the RNA samples, mixed and incubated at 37°C for 30min. A 0.1 volume of the DNase inactivation

reagent was added to the samples and incubated for 2min at room temperature. The DNase inactivation reagent was pelleted and left at the bottom of the tube.

2.2.4 Reverse Transcription PCR

2μg of DNase I treated RNA (see section 2.2.3) was added to a total volume of 10μl and incubated at 70°C for 10min to denature the RNA. 200ng of random primers (Gibco/BRL) was added to the reaction and chilled on ice. The following was added to the reaction mixture containing a final concentration of 1x first strand synthesis buffer (Gibco/BRL), 1mM each dATP, dCTP, dGTP, and dTTP (Gico/BRL), 2mM DTT, 200U of reverse transcriptase (Superscript II RNaseH$^-$ Reverse Transcriptase) (Gibco/BRL) in a final volume of 20μl. The cDNA synthesis reaction was carried out at room temperature for 10min, 42°C for 50min, and 70°C for 15min. The samples were cooled on ice. Typically, 1-2μl of the reaction was used as a template in subsequent PCR reactions.

2.2.5 Northern Analysis

RNA was fractionated on a 1.2% agarose/2.2 M formaldehyde gel. To prepare the gel, 3.6g of agarose and 216.3ml of distilled water were melted and cooled to 55°C. 53.7ml of formaldehyde and 30ml of 10X formaldehyde gel-running buffer (0.2M MOPS, pH7.0, 80mM NaOAc, 10mM EDTA, [pH8.0]) were pre-warmed to 55°C and added to the melted agarose. 20μl of 10μg/ml ethidium bromide was added to the solution before it was poured into a DNA sub cell gel kit (BioRad). When set, the gel was submerged in 1X formaldehyde gel-running buffer to cover the wells.

For each of the four RNA samples, 5.5μg of RNA in a total volume of 5.5μl, 1μl of 10X formaldehyde gel-running buffer, 3.5μl of formaldehyde, and 10μl of formamide were aliquoted into a 1.5ml microcentrifuge tube. 3μl of a 0.24-9.5Kb RNA ladder (Gibco BRL) were diluted in the same solution as the RNA samples. The RNA ladder and the RNA samples were incubated for 15min at 65°C and then cooled on ice. 2μl of

formaldehyde gel loading buffer (50% glycerol, 1mM EDTA, [pH8.0], 0.25% bromophenol blue) and 2µl of ethidium bromide were added to all the samples and these were loaded into the gel wells. The gel was electrophoresed overnight at 35V until the bromophenol blue had moved 8-11cm down the gel. Before blotting, the gel was washed with a solution containing 50mM NaOH, 150mM NaCl for 30min followed by a second wash with a solution containing 0.1M TRIS, [pH7.5], 150mM NaCl, for 30min. Finally the gel was rinsed with milli-Q water and then covered with 20X SSC (3M NaCl, 0.3M $Na_3Citrate.2H_2O$, [pH7.0]) for 45min.

RNA was transferred onto a nylon membrane by capillary action as described in section 2.1.22.

2.2.6 Northern Blots Probed with DNA

Prior to probing, the nylon membrane was pre-hybridised with a solution containing 5X SSPE (0.75M NaCl, 50mM $NaH_2PO_4.H_2O$, 5mM EDTA[pH7.4]), 5X Denhardts, 50% formamide, 0.5% SDS and 100µg/ml of denatured salmon sperm DNA (Sigma). The membrane and pre-hybridisation solution was incubated in a sealed plastic bag or in a hybridisation bottle for 4 hr at 45°C.

To hybridise the membrane with the DNA probe, the pre-hybridisation solution was replaced with a hybridisation solution containing 5X SSPE, 5X Denhardts, 50% formamide, 10% dextran sulphate, 5% SDS, 200µg/ml of denatured salmon sperm DNA and the labelled DNA probe. The membrane, hybridisation solution and labelled probe were incubated overnight at 45°C.

The membrane was subjected to a series of washes to remove non-specific binding at a high stringency. The first washes were 2 x 15min at room temperature with a solution containing 5X SSPE, 0.5% SDS. The second washes were 2 x 15min at 37°C with a solution containing 1X SSPE, 0.5% SDS. The final washes were 2 x 15min at 55°C with a solution containing 0.1 SSPE, 1% SDS.

Bound probe was detected by autoradiography as previously described.

2.3 Protein Expression and Purification Studies

2.3.1 Expression of pET-22b(+) Vector

One colony containing the pET-22b(+) vector with M4 insert was picked from a freshly streaked agar plate composed of LB, bacteriological agar and 50μg/ml of carbenecillin. The colony was inoculated into 10ml of LB and 50μg/ml of carbenecillin in a universal tube. The culture was incubated in the Orbital Shaker for 5 hours at 37°C until OD_{600} reached between 0.4 and 1. A 500μl aliquot of the culture was removed for the uninduced control and stored as the timepoints below. IPTG from a 100mM stock was added to the culture to a final concentration of 1mM and the incubation was continued for 3 hours.

To study the timecourse of target protein expression after induction, 500μl aliquots were saved at 60min timepoints following IPTG addition. The cells were collected by centrifugation and the supernatant was discarded. The cell pellet was resuspended in 50μl of SDS gel sample buffer (2% SDS, 4% glycerol, 0.125M Tris-HCL, [pH6.8], 0.004% bromophenol blue, 5% β-mercaptoethanol) and kept frozen at -20°C. When all the timepoints were collected, they (including the uninduced control) were ran on a SDS-polyacrylamide gel as below. The tube was placed on ice for 5 min and the cells were harvested by centrifugation at 500*g* for 5 min. The cells were resupended in 0.25 culture volume of cold 50 mM Tris-HCL [pH8.0] at 25°C and centrifuged as above. The supernatant was removed and the cells were stored as a frozen pellet at -70°C.

2.3.2 Expression of pGEX1 Vector

One colony containing the pGEX1 vector with M4 insert was picked from a freshly streaked agar plate composed of LB, bacteriological agar and 50μg/ml of carbenecillin.

The colony was inoculated into 10ml of LB and 50μg/ml of carbenecillin in a universal tube. The culture was incubated in the Orbital Shaker overnight and diluted 1 in 10 in 5ml of LB the next morning. The diluted culture was grown at 37°C until OD_{600} reached between 0.4 and 1. A 500μl aliquot of each culture was removed for the uninduced control and these cells were collected by centrifugation and the supernatant was discarded. The cell pellet was resuspended in 50μl of SDS gel sample buffer (2% SDS, 4% glycerol, 0.125M Tris-HCL, [pH6.8], 0.004% bromophenol blue, 5% β-mercaptoethanol) and kept frozen at -20°C. IPTG from a 100mM stock was added to the cultures to give a final concentration of 0.5, 1mM, and 1.5mM and the incubation was continued for 3 hours. 1.5ml of each culture was transferred to a sterile micro-centrifuge tube and centrifuged at low speed for 5min. The pellet was resuspended in 300μl of PBS, 1% Triton-X100 (Sigma), 1mM PMSF, and 1% Aprotinin. Cells were lysed by adding an equal volume of a 50% suspension of silica lysis beads/PBS to the resupended pellet contained in the micro-centrifuge tube. The tube was vortexed three times for 15sec and centrifuged at full speed. The supernatant was transferred to a fresh tube and the pellet was stored at -80°C. 10μl of sample buffer were added to 10μl of each supernatant and proteins were separated by SDS-PAGE. The excess supernatants were stored at -80°C.

2.3.3 Purification of GST Fusion Protein

Small scale purification of GST fusion protein for SDS-PAGE gels was as follows. 50μl of a suspension of Glutathione-Sepharose (Pharmacia Biotech) was equilibrated with four changes of PBS. The suspension was added to 500μl of supernatant collected from the protein expression of the pGEX1 vector with the M4 gene. This was incubated at room temperature for 5min then 1ml of PBS/1% Triton X100 was added. The tubes were centrifuged at a low speed for 30sec and the supernatant was carefully removed and discarded. The pellet was washed twice with PBS and then resuspended in 50μl of SDS-PAGE sample buffer. The pellet containing the silica beads stored from the protein

expression of pGEX1 vector procedure was also resuspended in 150µl of sample buffer. 10µl of each resuspension were loaded onto a SDS-PAGE gel.

Large scale purification of GST fusion protein for preparation of antigen for rabbits was performed using Glutathione-Sepharose (Pharmacia Biotech) according to manufacturer's instructions.

2.3.4 SDS-polyacrylamide Gel Electrophoresis (SDS-PAGE)

The bacterial cell lysates suspended in SDS gel sample buffer were heated for 5min at 95°C. If the gel was to be visualised by Coomasie Blue staining (see section 3.1.5), low molecular weight protein markers (Pharmacia Biotech) were used. If the gel was for western blotting "Rainbow" markers were used (Amersham). Both kinds of markers were also heated alongside the cell lysate samples. Samples were electrophoresed using a vertical slab gel system (Biorad). The main separating gel was composed of 15% acrylamide:bisacrylamide, 375mM Tris-HCl [pH8.7], 0.1% SDS, 0.7mg/ml ammonium persulphate, and 0.08% TEMED (Sigma). The stacking gel was composed of 5% acrylamide:bisacrylamide, 125mM Tris-HCl [pH6.8], 0.1% SDS, 0.8mg/ml ammonium persulphate and 0.2% TEMED. Gels were electrophoresed at 200V for 30 min or until the dyefront had reached the bottom of the gel.

2.3.5 Coomasie Blue Staining

Coomasie stain (0.25% Coomasie Blue R250, 10% acetic acid, 45% methanol) was used to visualise the proteins on the SDS-PAGE gel by incubating the gel in the stain for 30 min. The gel was destained by incubating in Coomasie destain (10% acetic acid, 20% methanol) overnight until the background cleared.

2.3.6 Western Blotting

3 layers of 3MM chromatography paper [16 x 16cm] (Whatman) were soaked in blotting buffer (48mM Tris, 39mM glycine, 20% methanol) and placed on a Semi-Dry Electroblotter (Ancos, Denmark). The nitrocellulose membrane (Sartorius AG,

Germany) was cut to the size of gel, soaked in blotting buffer and placed on the paper. The gel was removed from the plates, placed on the nitrocellulose membrane and covered with another 3 layers of 3MM paper soaked in blotting buffer. The electroblotter was electrophoresed at 120mA for 1 hour. The nitrocellulose blot was placed in 5% dried skimmed milk in PBS for 30min on a shaker, rinsed in PBS, and placed in monoclonal anti-polyHistidine antibody (Sigma) overnight at 4°C. The blot was washed in 5 changes of 1% dried milk in PBS over 30min. It was then placed in anti-mouse IgG biotin conjugate (Sigma) diluted with 1 % dried milk in PBS for 1 hour at room temperature with shaking. The blot was washed as before and incubated in streptavidin-alkaline phosphatase conjugate (Boehringer Mannheim) for up to 1 hour at room temperature with shaking. The blot was washed as before and was developed with alkaline phosphatase substrate tablets (Sigma). The process was stopped by rinsing the blot in water.

2.3.7 Purification of Anti-M4 Serum

Anti-M4 serum were purified using MAbTrap G (Pharmacia LKB) according to the manufacturer's instructions. The protein concentration of the purified anti-M4 sera was measured using the Bio-Rad Protein Assay as per manufacturer's instructions.

2.3.8 Analysis of Protein from Mammalian Cell Supernatant

His-bind resin (Novagen) was used to purify the expressed protein containing the His tag from COS-7 cell supernatant. 500µl of resin were added to 50ml of supernatant and centrifuged end over end at 4°C overnight. After removal of supernatant, the resins were boiled with SDS-PAGE sample buffer and proteins were analysed by western blotting with a monoclonal anti-His antibody (Sigma).

2.3.9 Immunoprecipitation

[^{35}S] labeled cell lysates and supernatants were precleared by incubating with 200µl sepharose 4B (Pharmacia) in RIPA buffer end over end overnight at 4 °C in screw capped microfuge tubes. 20µl of antibody was added to each sample incubated

overnight at 4 °C. 40µl of 50% (v/v) protein A sepharose (Sigma) in RIPA buffer was added to the samples and incubated end over end for 4 hours at 4°C. The protein A sepharose was centrifuged at low speed and the pellets were washed 5 X with 500µl of RIPA buffer. To analyse the precipitate, 40µl of sample buffer were added to each pellet and boiled for 2min at 100°C. The protein A sepharose was centrifuged and 50% of each sample buffer was loaded on a long 12% SDS-PAGE gel. The gel was electrophoresed overnight at 40V and Coomasie stained and destained as described in section 2.3.5. To reduce the exposure time a scintillant was incorporated into the gel which resulted in photon production by the radiation emitted from the isotope. The gel was submerged in En^{3}hance (DuPont), placed on a shaker for 30min. and rinsed in tap water for 30min. The gel was dried in a gel dryer (Biorad) and developed as described in 2.1.23.

2.4 Cell and Virus Culture

2.4.1 Tissue Culture

Baby hamster kidney (BHK-21) cells were propagated as a monolayer anchored to a T175 flask (Nunc). Cells were seeded at 2 x 10^6 cells with BHK growth medium (Glasgow modified Eagle medium (GMEM) (Gibco BRL) supplemented with 10% (v/v) tryptose phosphate broth (TPB) (Gibco BRL) and 10% new born calf serum (NBCS) (Harlan Sera Lab), 70µg/ml penicillin (Merck BDH), 10µg/ml streptomycin (Sigma) and 2mM L-glutamine (Merck BDH). Cells were incubated at 37°C in 5% CO_2 until confluent.

COS-7 cells were grown in with Dulbecco's modified Eagle medium (DMEM) (Gibco BRL) containing 10% Foetal calf serum (FCS) (Globepharm), 70µg/ml penicillin, 10µg/ml streptomycin and 2mM L-glutamine.

C127 cells were grown in T175 flasks (Nunc) seeded at 2 x 10^6 cells as for COS-7 cells.

293 cells were grown in T175 flasks (Nunc) seeded at 2 x 10^6 cells with DMEM containing 5% Foetal calf serum (FCS) (Globepharm), 70μg/ml penicillin, 10μg/ml streptomycin and 2mM L-glutamine.

Cell monolayers were harvested by washing with 10ml of versene solution (0.02% ethylenediaminetetra acetic acid (EDTA) and 1% phenol red, [pH 7.1-7.3] in sterile PBS) and then incubating with 8ml of Trypsin/EDTA solution (GibcoBRL) until monolayers peeled from the plastic surface. Cells were dislodged by tapping the flask and cell aggregates were broken by pipetting the cells up and down. 12ml of growth medium were added to the trypsinised cells, cell suspension were transferred to a universal tube and the cells were pelleted by centrifugation at 500*g* for 5min. The supernatant was discarded and the cells were resuspended in 20ml fresh medium ready to be seeded into flasks. The cells were vortexed for 10-20 sec to ensure complete resuspension. The appropriate volume of cells was added to a T175 flask and incubated at 37°C in 5% CO_2.

2.4.2 Mammalian Cell Quantification

The number of viable mammalian cells were quantified using a haemocytometer counting chamber. A 1:1 dilution of cells was prepared with 0.1% Trypan blue (0.1% trypan blue (Gurr) in PBS) and dispensed onto a well of the haemacytometer. The cells within the centre square and the four large corner squares were counted. Cells touching the upper and right hand perimeter lines were ignored and those touching the left hand and lower perimeter lines were counted. Viable cells appeared unstained whereas dead cells stained blue.

2.4.3 Storage and Retrieval of Cells

To store cell lines, cells were reuspended at 10^6 cells/ml in freezing solution (10% dimethyl sulfoxide (DMSO) in foetal calf serum). 1ml aliquots were transferred into

cryovials and frozen slowly in a –80°C freezer overnight. The cryovials were then transferred to liquid nitrogen tanks, -180 °C, for long term storage.

To retrieve cell lines, cells were thawed rapidly by placing the cryovial in a 37 °C water bath. The thawed cell suspension were transferred to a universal tube and 9ml of media were added drop by drop using a pasteur pipette, with constant swirling of the tube. Cells were centrifuged and transferred into a T25 flask (Nunc).

2.4.4 Electroporation of Cells

Cells for transfection are required to be ~80% confluent to increase the transfection efficiency. Cells were harvested by trypsinisation and resuspended at 2 x 10^6 cells/ml in their standard growth medium. 1ml of cells was transferred to a electroporation cuvette (Equibio). DNA to be transfected in a range of 5-15μg was added to the cells and mixed by pipetting. The cuvette was placed into the electroporator machine (Equibid) and transfected either using a single pulse (260V for 18msec, 1050μF capacitance) or a double pulse (first pulse 600V, 25mF capacitance, 99Ω, 0.1s delay, second pulse, as single pulse). Electroporated cells were resuspended immediately in 10ml medium, split between 2 wells of a 6 well plate (Nunc) and incubated in a 5% CO_2 incubator at 37°C. The medium covering the wells was changed after 24 hours and selective agents applied if required.

2.4.5 Generation of a Stable Mammalian Cell Line

To generate a stable cell line expressing M4 protein, three cuvettes containing
(i) COS-7 cells and 5μg pcDNA3.1/V5/His TOPO vector (Invitrogen) with the M4 gene, (ii) COS-7 cells and 5μg pcDNA3.1/V5/His TOPO vector only, (iii) COS-7 cells only, were transfected using a single pulse. Cells were incubated in a 5% CO_2 incubator at 37°C for 3 days. Cells were harvested and resuspended in 10ml of medium containing Geneticin (G418 Sulphate) (GibcoBRL) at 400μg/ml. Cells were plated into 6-well plates and replaced with selective medium twice a week until foci could be identified.

After four weeks the antibiotic resistant cell foci were ring cloned. Ring cloning involved removing the medium from the well and placing a sterile ring (cut from the base of a Gilson pipette tip) with a coating of silicon grease around a focus of cells. Cells were harvested by washing with versene and trypsinisation. Cells were transferred into a well of a 6-well plate and grown in selective medium. Confluent cells were harvested and grown in T25 flasks.

2.4.6 Transfection of M4KO DNA and MHV-68

To generate a M4KO virus, four cuvettes containing BHK cells and different ratios of M4KO cassette to MHV-68 DNA were set up as follows:, (i) 15μg: 10μg, (ii) 10μg: 10μg, (iii) 1μg: 10μg, (iv) no DNA were electroporated using a single pulse. Transfected cells were incubated in a 5% CO_2 incubator at 37°C overnight. Cells were rinsed with medium and overlayed with 5ml of agar (2% Seakem plaque agarose (Flowgen) melted in PBS mixed with an equal volume of medium). Cells were incubated in a 5% CO_2 incubator at 37°C.

2.4.7 Transfection of M4KI DNA and MHV-76

To generate a M4KI virus, four cuvettes containing BHK cells and different ratios of M4KI cassette to MHV-76 DNA were set up as follows, (i) 10μg: 5μg, (ii) 10μg: 10μg, (iii) 5μg: 0μg, (iv) no DNA were electroporated using a double pulse. Transfected cells were incubated in a 5% CO_2 incubator at 37°C overnight. Cells were rinsed with medium and overlayed with 5ml of agar (2% Seakem plaque agarose (Flowgen) melted in PBS mixed with an equal volume of medium). Cells were incubated in a 5% CO_2 incubator at 37°C.

2.4.8 Radiolabelling Cells with [^{35}S] Methionine

BHK cells which were sub-confluent and growing exponentially in 6 well plates were infected with 5-10 pfu/cell of MHV-68 virus in 500μl medium or mock-infected. After 1 hour at 37°C the virus or mock-infecting medium was removed and 2ml of fresh

medium were added to each well. At 4 hour post-infection the medium was removed and cells were rinsed in medium containing 20% normal methionine and 2% serum (10mls GMEM, 40ml methionine and serum free medium (Gibco BRL), 2mM L-glutamine). 200μCi [^{35}S] methionine (Amersham) were added to each well in a total volume of 1ml. Wells were incubated overnight at 37°C. Supernatants were removed from each well and stored. Cells were harvested in 1ml RIPA buffer (20 mM Tris, [pH7.2], 0.15 M NaCl, 1% (v/v) Triton X100 (Sigma), 1% (w/v) deoxycholate, 0.1% (w/v) SDS). Both cell lysates and supernatants were kept at -80 °C and used for immunoprecipitation.

2.4.9 Preparation of Mouse Splenic Lymphocytes for MACS

The spleen was placed in a 60mm Petri dish containing 5ml of RPMI 1640 medium (Gibco BRL) supplemented with 10% FCS, 70μg/ml penicillin, 10μg/ml streptomycin, 50μM β-mercaptoethanol (ME) (Sigma) and 2mM L-glutamine. Cells were teased out using a sterile scalpel and transferred to a universal. Another 5ml of media were used to rinse the remaining cells from the Petri dish. Cells were centrifuged for 5min at 500*g*, and resuspended in 10ml of media. After cell clumps had sedimented, the single cell suspension was removed by decanting, layered onto 10ml of histopaque (Sigma) and centrifuged for 20min at 3000*g* with no brake. The lymphocytes were removed from the interface and transferred to a universal containing 15ml of media. They were centrifuged for 10min at 900*g*, resuspended in 20ml of media and centrifuged for a further 10min at 500*g*. After resuspension in 20ml of media, lymphocytes were counted using a haemocytometer.

The MACS (Miltenyi Biotec) column was prepared (see below) while lymphocytes were washed and counted. Cells were washed with ice cold 1% FCS in PBS and resuspended in the same buffer at 5x10^{7}cells/450μl. 60μl of CD43 microbeads (Miltenyi Biotec) were added to 5x10^{7}cells and incubated for 15min in a 4 °C cold room or fridge.

Meanwhile the column was prepared. The column was placed by the magnet and filled with 70% alcohol using the 3 way tap and a syringe which filled from bottom up. A 23 gauge needle was fitted onto the column and the ethanol was allowed to flow through. The column was washed with at least 15ml of ice cold 1%FCS/PBS and kept full of buffer until it was ready for use.

15ml of 1%FCS/PBS were added to the CD43 microbeads and cells and centrifuged for 5mins at 500*g*, 4°C. Cells and beads were resuspended in 1ml of 1%FCS/PBS and loaded onto the column. The flow through were collected and the column was washed with 15ml of ice cold 1%FCS/PBS. The flow through were centrifuged, resuspended in medium and counted.

2.4.10 Alamar Blue Assay

$2x10^5$ cells were added per well of a 96 well flat bottomed plate (Iwaki). AlamarBlue (Serotec) was added to each well to a final concentration of 10% (v/v). Different concentrations of supernatants were added to triplicates of wells containing cells. The absorbance of the cultures was measured at a wavelength of 570nm and the background absorbance measured at 600nm was subtracted from this reading. The plates were incubated at 37 °C in a CO_2 incubator for 3 days with absorbance measurements taken using a Dynatech MR 5000 (Dynex) every 24 hours.

2.4.11 [^{3}H] Thymidine Assay

100µl of a cell suspension containing $2x10^5$ cells was aliquoted into a total volume of 200µl in a 96 well flat bottomed plate (Iwaki). Different concentrations of supernatants were added to triplicates of wells containing B cells. The plates were incubated at 37 °C in a CO_2 incubator. After 48 hours, 1µCi of [^{3}H] thymidine was added to each well and incubation was continued for 24 hours in a CO_2 incubator. Cells were harvested onto filtermats using a cell harvester (TOMTEK). The incorporated radioactivity of each cell sample was read using a 1450 Microbeta liquid scintillation counter (Wallac).

2.4.12 MHV-68 Stock Preparation

BHK-21 cells were grown up to the numbers required, trypsinised, and resuspended to 10^7 cells/ml in a 50 ml tube. MHV-68 was added to the cells at a multiplicity of infection (MOI) of 0.001 pfu/cell. The tube was incubated with shaking for 1 hour at 37°C. The cells were seeded at $3x10^6$ cells/flask and incubated for 4-5 days in 5% CO_2 at 37°C until 100% cytopathic effect was observed. A cell scraper was used to remove any remaining cells from the plastic surface of the flasks. The media was pipetted into 50ml falcon tubes. The flasks were rinsed with a very small amount of media to ensure all cell debris was removed. The 50ml tubes were centrifuged at 2000*g* for 20min at 4°C and the supernatant was removed. A total of 5-6ml of ice cold PBS were used to resuspend all the cells. Resuspended cells were pipetted into a Dounce homogeniser (Wheaton) which was used to disrupt the BHK cells. Cells in the homogeniser were transferred to a 50ml tube and the tube was centrifuged at 2000*g* for 20min at 4°C. The supernatant was removed and kept on ice. The pellet was resuspended in 1ml of ice cold PBS, homogenised and centrifuged again as above. The second supernatant was pooled with the first supernatant. The pellet was stored at - 80°C until the virus stock was titrated in the supernatant. The supernatant was dispensed into 0.2 and 0.5ml volumes and stored at -80°C. Infective virus was measured by plaque titration using BHK-21 cells.

2.4.13 Plaque Purification of Recombinant Viruses

Cells which were infected with virus and had undergone homologous recombination with the recombinant DNA cassettes formed plaques expressing the green fluorescent protein (GFP). Green plaques were visible by fluorescence microscopy using a UV filter (excitation at 450nm). Pasteur pipettes were used to pick plaques by piercing the agar and removing the agar directly above the plaque of interest. Agar containing the plaque was dispensed into 1ml of medium, sonicated x 3 in a sonicating water bath for about 20sec and freeze/thawed to disrupt the cells

To purify the green virus from wild-type, the picked plaque suspensions were serially

diluted from 10^{-1} to 10^{-6}. 1 x 10^6 C127 cells were added and tubes were incubated for 1hr at 37 °C with shaking to allow adsorption. Cells were aliquoted into a well of a 6 well plate and incubated in a 5% CO_2 incubator at 37°C. After 24 hours, the media on the monolayer was replaced with an agar overlay. After 3-5 days, green plaques were picked and the dilution procedure repeated. This process was repeated at least 3 times.

2.4.14 Infection of Cells for RNA Transcript Analysis

Four T175 flasks containing BHK-21 cells at 10^7 cells/flask grown up in Glasgow's media were used. To distinguish immediate early and early RNA transcribed post-infection, cyclohexamide and the antiviral compound 2'deoxy-5'ethylβ-4'thiouridine (4'-S-EtdU) (GlaxoWellcome Laboratories, Kent) (Rahim *et al.*, 1996) were used respectively. The medium from one flask was decanted and 10ml of Glasgow's media containing 100μg/ml of cyclohexamide was added to cover the cells prior to infection. This flask set up for immediate early RNA was incubated for 30mins with 5% CO_2 at 37°C, and then the solution was removed. The flasks set up for immediate early, early, and late RNA were infected with MHV-68 at $5x10^7$ pfu/cell in 5ml of Glasgow's media containing 100μg/ml of cyclohexamide, 2μg/ml of 4'-S-Etdu and only virus, respectively. The fourth flask was used for mock infection and contained only 5 ml of Glasgow's media. All the flasks were incubated with 5% CO_2 at 37°C. After 1hr, the Glasgow's media were increased in all 4 flasks to a total of 30 ml/flask containing their respective inhibitors. 8hr post-infection, the flask containing the immediate early RNA was harvested. The flasks containing the early RNA, late RNA and mock infected cells were harvested 24hr post-infection. To harvest the RNA, the media in all flasks was decanted and the cells covering the plastic surface were carefully washed with 20ml of ice cold PBS/flask. 3ml of RNAzol B (Sigma) were added to each flask to remove the cells from the plastic surface. The cell homogenates were transferred to 4 separate universal tubes.

2.4.15 Mice

BALB/c females were used, age 3 to 4 weeks on arrival from B & K Universal Ltd (Grimston, Aldborough, Hull, UK).

2.4.16 Administration of Virus to Mice via Intranasal Route

Mice were inoculated intranasally with 4 x 10^5 pfu of virus in a 40µl infusion. Prior to administration mice were lightly anaesthetised using halothane. Virus dilutions were made in sterile phosphate buffered saline immediately prior to use and were kept on ice until the inoculation.

2.4.17 Sampling of Mice

Mice were killed in a CO_2 chamber and relevant organs removed by dissection.

2.4.18 Assay for Infective Virus

If required, sample organs were allowed to thaw and then homogenised individually in 1ml of ice cold supplemented GMEM using glass homogenisers. Cell suspensions were transferred back to their cryovials and refrozen at -70^0C. Freeze/thawing samples increases cell lysis and infective virus release.

Tissue homogenates were thawed rapidly at 37^0C and centrifuged at 1100*g* for 5 minutes at 4^0C to pellet cell debris. Homogenates or virus samples were then diluted in a bijou and $2x10^6$ BHK-21 cells added. The samples, in a final volume of 4ml of supplemented GMEM, were incubated at 37^0C for one hour on a shaker, to allow virus adsorption. After incubation, the samples were vortexed, then the volume split between two 60mm Petri dishes containing 2ml supplemented GMEM to form duplicates. The suspensions were gently swirled to evenly mix and distribute the cells. All Petri dishes were subsequently incubated at 37^0C with 5% CO_2 for 4 days. Following this incubation, the BHK cell monolayers were fixed by removing supernatant from the plates using a 25ml pipette and replacing it with 3ml of 10% formal saline. The plates were then incubated for a minimum of 15 min at room temperature. In a fume hood, the formal saline was

poured off and around 5ml of 0.1% toluidine blue was added to each plate to stain the monolayer. After 20 min the stain was poured off and the plates were rinsed in water and allowed to air dry.

The number of plaques per plate was quantitated using a microscope and the virus titre per organ was calculated.

2.4.19 Assay for Infective Centres

Splenocyte suspensions were obtained and counted as previously described. 1 x 10^7, 1 x 10^6 and 1 x 10^5 cells were pipetted in duplicate into 60mm Petri dishes containing 6ml of RPMI medium and 1 x 10^6 BHK-21 cells. 1ml of each splenocyte suspension was refrozen for subsequent infectious virus titration. Plates were incubated for 5 days at 37^0C with 5%CO_2. After this period monolayers were fixed as described above. Numbers of infective centres/plate was quantitated using a microscope and the infective centre titre was calculated per 10^7 splenocytes and per spleen.

2.4.20 One Step Growth Curve of Virus

BHK-21 cells at 80% confluency in a 24 well plate were infected with virus at 5 pfu/cell and allowed to absorb for 1 hour at 37°C in 5% CO_2 incubator. Unbound virus was removed from the wells, each well was washed three times with medium and 1.5ml of medium were aliquoted into each well. The 0 hour timepoint was harvested in duplicate and stored at -70 °C and the 24 well plates were incubated at 37°C in 5% CO_2 incubator. Thereafter, wells were harvested in duplicate at appropriate timepoints and stored at -70 °C. The virus was released from the cells by freeze/thawing at -70 °C three times and titrated as described in section 2.4.19.

2.4.21 Histopathology

After euthanasia of mice by CO_2 asphyxiation, the lungs were perfused *in situ* via the trachea with 10% neutral buffered formal saline. Portions of the spleen were also fixed in 10% buffered formal saline and the tissues were processed routinely into paraffin

wax-embedded sections. These were stained with hematoxylin and eosin, and examined by light microscopy.

2.5 Immunological Studies

2.5.1 Flow Cytometric Analysis of Cells

Reagents:

1% formal-saline – 10% formal-saline solution diluted 1/10 v/v with sterile PBS

FACS buffer – PBS with 1% BSA and 0.1% Sodium Azide (w/v)

All antibody working stocks diluted in FACS buffer

2.5.2 Immunostaining and Flow Cytometry

Flow cytometric analyses were carried out on splenocytes prepared or cultured as previously described. 1 x 10^6 cells were pipetted into micro-test tubes and the cells were pelleted at 370*g* for 5 min at 4^0C. The cells were re-suspended by agitation and 30µl of primary antibody was added to the appropriate tubes which were then incubated for 20 minutes on ice. Following incubation the cells were pelleted by centrifugation as above and the supernatants were removed by aspiration. Each aliquot of cells was washed 3 times with ice-cold FACS buffer. 200µl of the buffer was added to each tube containing a re-suspended cell pellet and the cells were re-pelleted by centrifugation. 30µl of secondary antibody was added to the cells if required. Following addition of the secondary antibody, the cells were incubated on ice for 20 min and then washed as before. Finally, the cells were fixed by addition of 200µl of 1% formal-saline to all tubes and analysed using a FACStar (Becton Dickinson) FACS scanner.

2.5.3 Immunofluorescence

BHK cells were infected overnight with MHV-68 at a multiplicity of infection (MOI) of 10 and plated into a 6 well plate. The cells were resuspended at 10^6/ml and 200µl aliquots were spread onto biobond slides using a cytocentrifuge. Slides were washed

with PBS for 2 min and blocked with 100µl of 5% pig serum in PBS/slide overnight. Slides were washed in PBS + 0.05% Tween 20 and then washed again in PBS alone. The primary antibody, rabbit anti-M4, were diluted in PBS + 2% pig serum and 100µl were added to the cells in 1:50, 1:100 and 1:500 dilutions. The cells were incubated for 1hr at 37°C and washed x 3 in PBS + 0.05% Tween 20. The secondary antibody, FITC-labelled swine anti-rabbit was diluted to 1:50 in PBS + 2% pig serum and 100µl were added to each slide. The cells were incubated for 1hr at 37°C and washed x 3 in PBS + 0.05% Tween 20. A drop of citifluor were added to each coverslip and used to cover each slide.

Chapter Three:

Expression Studies

3.1 Expression Results

When first provided with the opportunity to characterise and explore the function of a novel gene, an initial aim would be protein expression. M4 is a novel gene which has never been characterised and expressed in an expression system. Protein expression would allow for the generation of antisera which would be an invaluable tool in both *in vitro* and *in vivo* immunostaining studies. RNA expression studies would provide vital clues to the function of this gene as it would provide an indication of the time post-infection of when M4 is transcribed and whether it can be termed an immediate early, early, late or a latency gene.

This has been successfully demonstrated with many novel genes including a HHV-6 candidate for a chemokine homolog, U83 (Zou *et al.*, 1999). The U83 gene was cloned, expressed in a bacterial expression system and the purified protein was used to generate antisera. Immunostaining studies with the antisera revealed the expression pattern of U83 in infected cells and additionally demonstrated that U83 is a late gene (Zou *et al.*, 1999). M3, a putative chemokine of MHV-68 was initially characterised by northern blot analysis which revealed M3 is an early-late gene (Simas *et al.*, 1999, van Berkel *et al.*, 1999). RNA expression studies have also revealed which stage of viral infection other MHV-68 genes are expressed, including ORF 50 which was identified as an immediate-early gene (Liu *et al.*, 2000).

3.1.1 Analysis of the M4 Peptide Sequence

The DNA sequence of the M4 gene revealed no homology to known proteins (Virgin *et al.*, 1997). To further analyse the M4 gene for potential motifs and domains that may provide a clue to the function of the gene, the M4 sequence was translated from base position 8409 to 9785 into its corresponding 459 amino acids using the University of Wisconsin Genetics Computer Group Sequence Analysis Software (UWGCG 9.0, Devereux *et al.*, 1984) (see figure 3.1.1a). Seven potential N-glycosylation sites and a heparin sulphate binding domain were identified in the half of the peptide nearest the C-terminus. A hydrophobicity plot of the amino acid sequence was generated which

revealed a signal sequence at the N-terminus of the M4 gene (figure 3.1.1b). The signal sequence indicates that the M4 protein is secreted. Heparin sulphate binding domains are found in many cytokines, this indicates that the M4 protein may function as a potential cytokine (Handin & Cohen, 1976, Webb *et al.*, 1993).

3.1.2 M4 Protein Expression Using the pET System

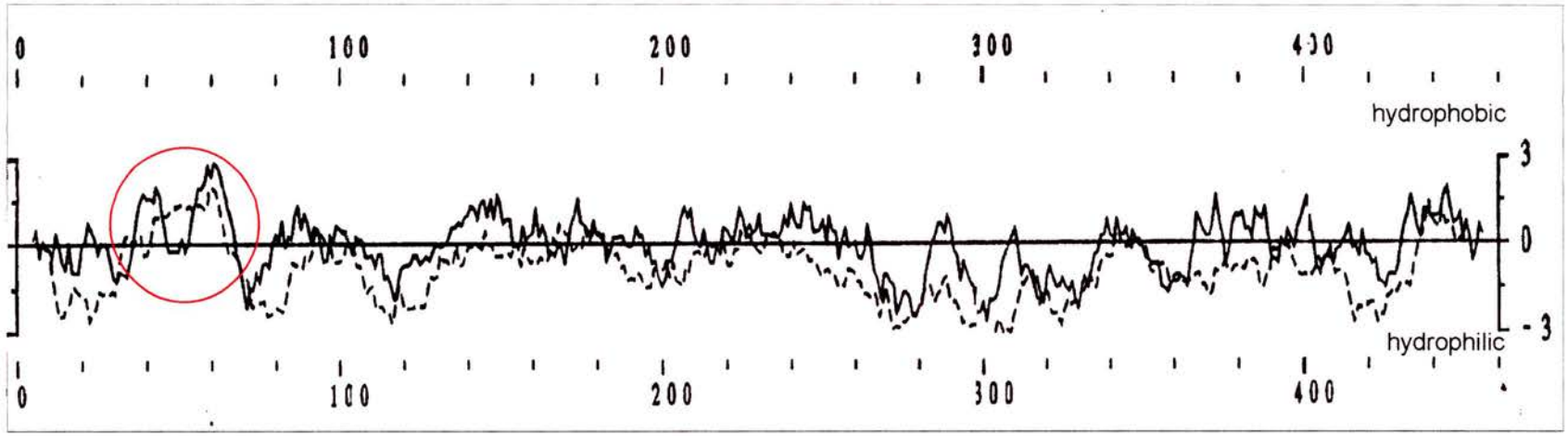
The pET-22b(+) vector was chosen to express the M4 protein because the system allows stringent control of expression. The target gene is inserted under the control of the bacteriophage T7 promoter and is cloned using hosts that do not contain the T7 RNA polymerase gene, thereby eliminating the possibility of plasmid instability due to the production of proteins which are toxic to the host. Expression of the gene is induced by providing a source of T7 RNA polymerase in the host cell. Futhermore, the vector contains a hexahistidine tag which is expressed in the C-terminus of the target protein allowing for detection and purification. The M4 gene was amplified via PCR (as described in 2.1.12) using primers M4A and M4B to produce a PCR product of 1.3kb in length. The M4 product was ligated into the pET-22b(+) vector using the *EcoR*I and *BamH*I restriction enzyme sites designed within the primers. The ligated vector was transformed into BL21(DE3) bacteria using the colony transformation procedure as described in section 2.1.9. Once transformed colonies were shown by restriction enzyme digest to be positive for the M4 gene, the pET-22b(+) vector was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) which induces T7 RNA polymerase to transcribe the gene as described in section 2.3.1. The proteins in induced whole cell lysates were visualised using Coomasie-stained SDS-PAGE gel, however, no induced protein was observed. To eliminate the possibility that the protein was present but in minimal amounts such that it was not visible, a more sensitive technique was used. Western blotting was performed as described in section 2.3.6 using two different antibodies, rabbit anti-MHV-68 polyclonal anitibody and mouse anti-his monoclonal antibody, as detecting agents (see figure 3.1.2). Normal rabbit serum and a control monoclonal antibody were used as negative controls and a pET-22b(+) vector containing the gp150 gene (provided by J.P. Stewart) was used as a positive control. Also, different

Figure 3.1.1a Amino acid sequence of the M4 gene.

1	MVGTQPRFLV	KIHQKFYWAI	RDDLIIHGGY	KRSGLTSSFA	LVAMGPLGRP
51	WAASFGFFFL	AVVSLATPTP	GEDDDIPVKI	HRLTFVKGLV	PDVTGGSSYY
101	VCVYPSRKLF	CTPTRWKDLS	RFLNSETLNQ	VCSARTIYSV	VPVEMLRIIS
151	LPLPPEIKSI	VGGSIRSQYV	SFPTFSETLNQ	AKLPIWKSFA	NLKVSMKFRS
201	SQWEVAFSVV	SKTDYAITYW	AEIPGFLIHE	SATINLINQP	LLALYADLHV
251	DMVMRLTDKF	IYCQTYTLQQ	KNLTDPRTGK	RPTSSVLIPS	PHVKNCQIRR
301	RNETHFVDTC	SSAWDNYTSE	AHNISRNSSS	RGSNATQLVN	ITANPCTLPT
351	LWDNWPCYTN	YRSSPVPEIV	IHENILLEGR	AIYIIYHQIG	LFDQPRLCVA
401	TFWMSKEETL	LMQLDYPCEV	SVEKKGKKFF	IKSVVSMYHA	ISMVTFIWEY
451	GIEIYDFLE				

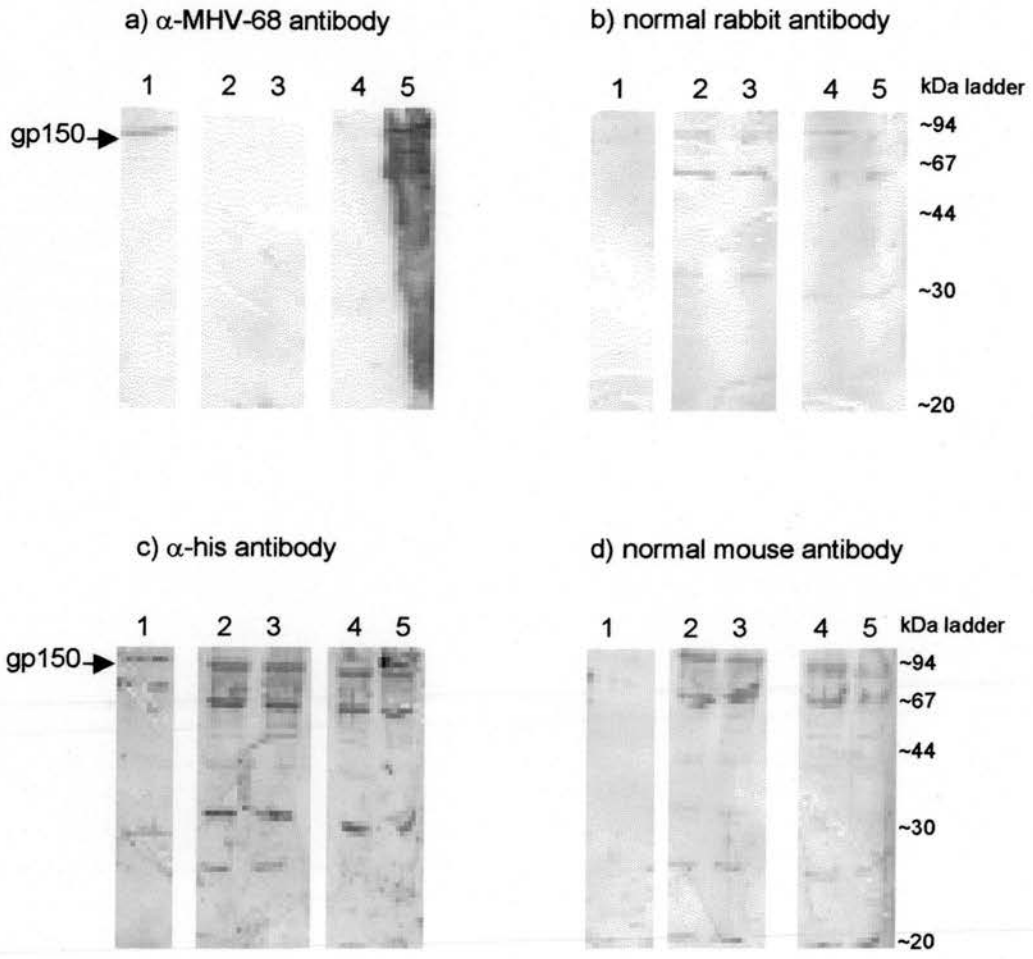
The M4 DNA sequence, base position 8409 to 9785, was translated into 459 amino acids. The 7 predicted N-glycosylation sites are shown in green letters. The heparin sulphate binding domain is shown in red letters.

Figure 3.1.1b Hydrophobicity plot of M4 peptide sequence.



Red circle indicates the predicted signal sequence .

Figure 3.1.2 M4 protein expression from pET-22b (+) vector.



Lane 1: purified gp150 protein

Lane 2: M4/pET-22b(+)in BL21(DE3), -IPTG

Lane 3: M4/pET-22b(+)in BL21(DE3), +IPTG

Lane 4: gp150/pET-22b(+)in BL21(DE3), -IPTG

Lane 5: gp150/pET-22b(+)in BL21(DE3), +IPTG

M4 gene was cloned into pET-22b(+) vector and induced with isopropyl-β-D-thiogalactopyranoside (IPTG) in BL21(DE3) cells. Whole cell lysates were electrophoresed on a 12% SDS-PAGE gel and western blotted with a) α-MHV-68 antibody, b) normal rabbit antibody, c) α-his antibody and d) normal mouse antibody. pET-22b(+) vector containing the MHV-68 gp150 gene and purified gp150 protein were used as controls.

dilutions of both antibodies and conjugates were used for the western blotting (not shown in figure 3.1.2). No M4 protein expression was visualised by western blotting. Both rabbit anti-MHV-68 and mouse anti-his antibodies bound to the gp150 protein which demonstrated that recombinant protein was being induced and that his tagged protein could be detected by the anti-his antibody. This also demonstrated that the anti-MHV-68 antibody was detecting antigens.

A further explanation for the lack of M4 protein detected was that the protein was expressed and present but in a very low amount such that it was undetectable by SDS-PAGE gel and western blotting. Therefore, the small scale purification method as described in the pET system manual (Novagen) was performed on induced whole cell lysates. However, no recombinant protein could be detected by western blotting.

Another possible reason for lack of M4 expression would be the introduction of a mutation into M4 during the PCR amplification and cloning. Three clones of the M4 DNA contained in the pET-22b(+) vector were sequenced for mutations. A T7 primer was used and the first 500 bases of the gene was sequenced. The sequenced stretch of DNA of one M4 clone was aligned with the M4 DNA sequence using the University of Wisconsin Genetics Computer Group Sequence Analysis Software (UWGCG 9.0, Devereux *et al.*, 1984). Accurate sequence of the first 500 bp of the M4 gene was obtained which revealed that no mutations were present. While it was possible that mutations had occurred further downstream, these clones should still have produced a product. The likelihood that a mutation accounted for the lack of protein was therefore small because a) three individual clones were tested for expression and it was unlikely that all three had mutations, and b) sequencing showed all three clones were intact up to 500 bp.

Another explanation was that low amounts of M4 protein expression may have been transcribed before the induction, and this may be toxic to the BL21(DE3) bacteria. This is caused by low levels of T7 polymerase being produced even in the absence of IPTG.

A final possible explanation for the lack of M4 protein expression was that ampicillin selection used during the induction procedure was being lost in the culture and bacteria had arisen which lack the M4 gene and have overgrown the bacteria which did contain the gene. Therefore, when the induction procedure was initiated, there were few bacteria in the culture which contained the pET-22b(+) vector and the M4 gene.

To overcome the problem of toxicity, another strain of bacteria which provides additional stability to target genes, termed BL21(DE3)pLysS were used. This strain contains a plasmid that encodes a small amount of T7 lysozyme which inhibits T7 RNA polymerase (Moffatt & Studier *et al.*, 1987, Studier, 1991). The BL21(DE3)pLysS bacteria were transformed and induced as before but this time the selective antibiotic was changed to carbenecillin. Carbenecillin, like ampicillin, is degraded by β-lactamase which is encoded by a gene within the pET-22b(+) vector. However, this antibiotic is degraded much slower and is more stable in cultures by being less sensitive to low pH. Therefore, in place of ampicillin, carbenecillin helps prevent overgrowth of culture with bacteria which lack the M4 gene-containing plasmid. Once again, western blotting with rabbit anti-MHV-68 and mouse anti-His antibodies did not detect M4 protein expression. In another strategy to try and overcome the potential toxicity of the M4 gene, the BL21(DE3)pLysS bacteria were grown up in a culture containing a higher concentration of carbenecillin and the medium was replaced twice prior to induction. During the induction, the culture was incubated at 30°C instead of 37°C. Even with these changes to the induction protocol no M4 protein expression was detected by western blotting.

3.1.3 M4A and M4B Protein Expression Using the pET System

Since we were unable to express the whole of the M4 protein, we decided to split the 1.3kb M4 gene in half and attempt to express the two separate fractions. Therefore, primers were designed and used to amplify the two fragments. The first half of the gene from nucleotide 8617-9176 nearest the 5' end, termed M4A, were amplified via PCR using primers M4A and M4ARev to produce a PCR fragment of 559bp. The second half

of the gene from nucleotide 9156-9786 nearest the 3' end, termed M4B, were amplified via PCR using primers M4BFor and M4B to produce a PCR fragment of 630bp (figure 3.1.3). The two halves of the M4 gene were cloned into the pET-22b(+) vector, re-transformed into the BL21(DE3)pLysS bacteria and induced. Again Coomasie-stained SDS-PAGE gels and western blotting with mouse anti-his antibody and rabbit anti-MHV-68 antibody did not detect any induced protein.

Once more the M4A and M4B DNA contained within the pET-22b(+) vector was sequenced to check for mutations. Accurate sequences were produced of the first 342bp and 502bp of M4A and M4B, respectively.

3.1.4 M4A and M4B Protein Expression Using the pGEX System

pGEX1 is a fusion-protein expression vector that expresses the cloned gene as a fusion protein to glutathione-S-transferase (GST). The GST protein acts as a control as it can be detected as a 27kD band on a SDS-PAGE gel. This new approach was used to express the M4 protein. The procedure involved the re-cloning of M4A and M4B into a different expression vector termed pGEX1. The M4A and M4B-containing pGEX1 vectors were transformed into XL-2 ultracompetent bacteria (Stratagene) and were induced. Coomasie-stained SDS-PAGE gel detected induced protein bands in the 50kD region for M4A whole cell lysates (not shown). The expression of the M4A protein was confirmed by western blotting using rabbit anti-GST antibody as shown in figure 3.1.4. The predicted sizes of the M4A and M4B proteins are 20kD, thus the protein bound to the 27kD GST would appear at a molecular weight of 47kD. In the case of M4B, we were unable to detect an induced band in the 47kD region by western blotting using rabbit anti-GST antibody.

3.1.5 Generation of Antisera

The M4A protein was purified on large scale and used to generate antisera in two rabbits, termed IG54 and IF55. The rabbits were immunised via a subcutaneous injection. The initial inoculation contained 20mg of M4A protein purified using the

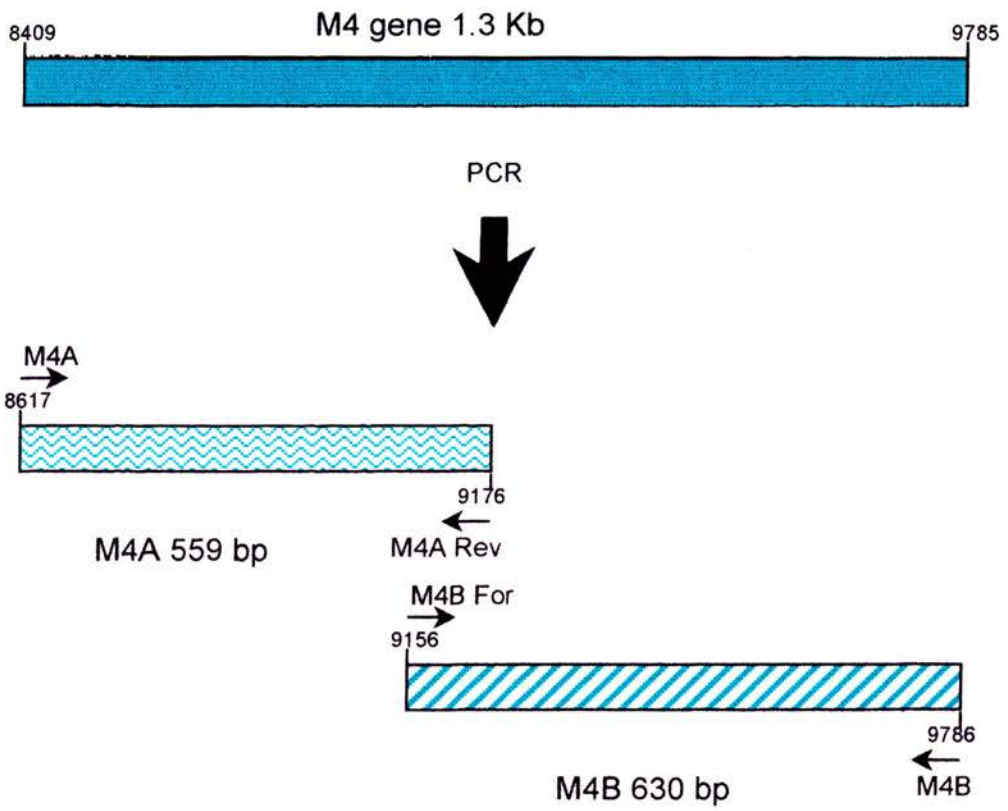
GST sepharose column (see section 2.3.3) homogenised with 1ml of TiterMax Gold (CytRx Corporation) and this solution was equally split between the two rabbits. Both rabbits were boosted three times with four week intervals. Each boost consisted of 10mg of M4A protein per rabbit. After four months from the initial inoculation, serum was obtained from blood samples from the rabbits and tested for anti-M4 activity.

3.1.6 Anti-M4 Activity of Antisera

The antisera was used to detect the M4A protein expressed in the pGEX system by western blotting (as described in section 2.3.6). A variety of different concentrations of sera, including neat sera, was used. The antisera bound to the M4A fusion protein, however, we were unable to show that this was due to M4 and not GST recognition (not shown).

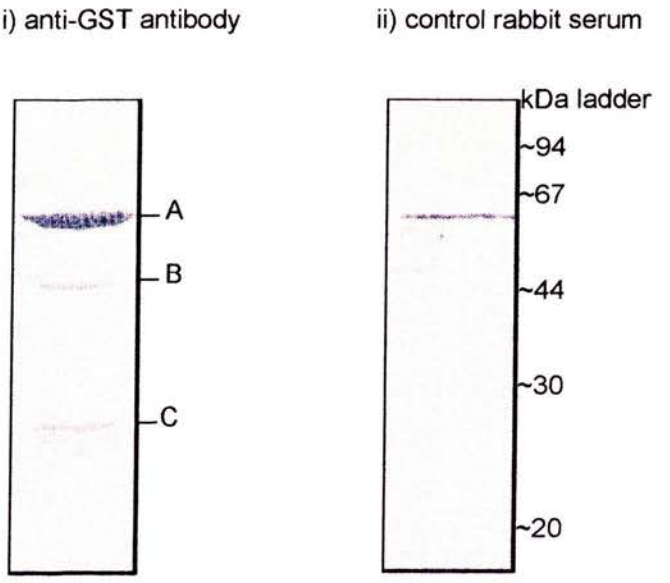
An immunoprecipitation approach (as described in section 2.3.9) was used as this was likely to be more sensitive. The late proteins of MHV-68 infected BHK cells were labelled with [^{35}S] methionine and both the pre-immune rabbit serum and anti-M4 rabbit serum were used for immunoprecipitation with the cell lysate and cell supernatant. The immunoprecipitated proteins were separated on a SDS-PAGE gel as shown in figure 3.1.6. The immunoprecipitation revealed that the anti-M4 sera bound to a protein in the range of 46kD in the lane containing the infected cell lysate. This observation confirms the predicted size of the 44kD M4 protein. In the same lane, the anti-M4 sera also bound to two unknown bands of ~80kD and ~94kD not bound by the pre-immune anti-sera. In the lane containing the infected cell supernatant, the anti-M4 sera precipitated a diffuse band of ~60kD. No proteins of these sizes were precipitated from the mock-infected cell lysates and supernatant. These studies show that the anti-serum bound specifically to proteins produced following MHV-68 infection. The intracellular 46kD protein is the expected size for M4 and is likely to be the M4 protein. The extracellular 60kD protein is likely to be a processed form. The "fuzzy" appearance suggests that it is processed possibly by the addition of sugars. The 80kD and 94kD proteins may be dimers but at present this is unknown.

Figure 3.1.3 Generation of M4A and M4B DNA fragments.



The M4 gene cut to generate two DNA fragments; M4A and M4B, using PCR with four primers (black arrows).

Figure 3.1.4 M4A protein expression.



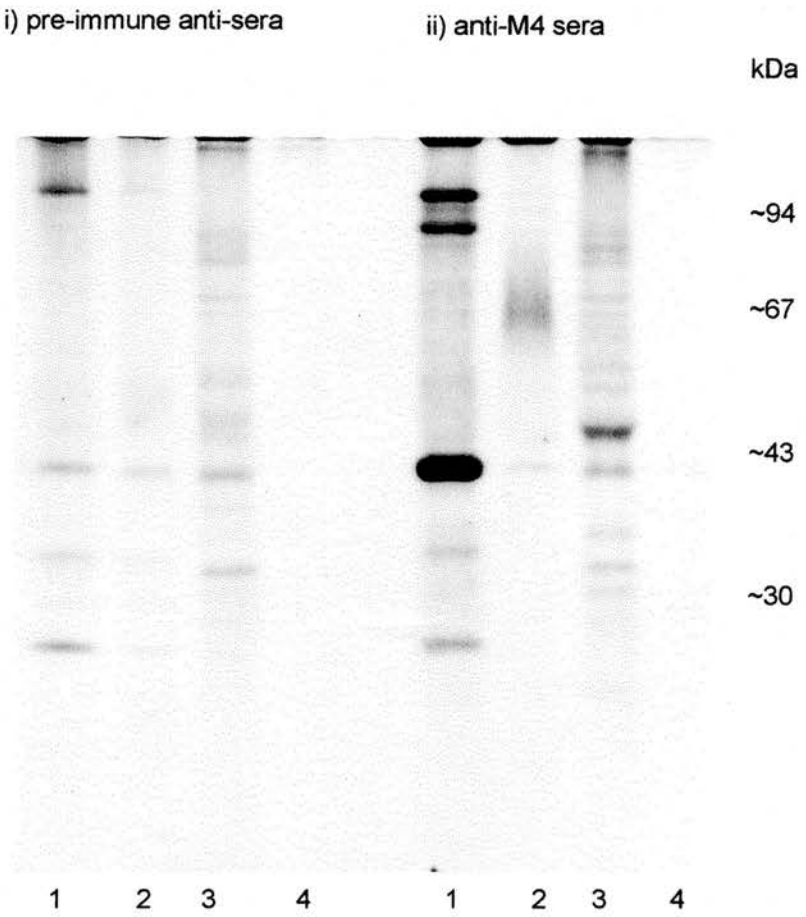
A. GST dimer, 60 kDa

B. M4A fusion protein, 46 kDa

C. GST monomer, 27 kDa

M4A gene was cloned and expressed in the pGEX-1 vector. The M4A protein was bound to GST beads and eluted with glutathione. The eluted protein was electrophoresed on a 12% SDS-PAGE gel and western blotted with i) anti-GST antibody and ii) control rabbit serum.

Figure 3.1.6. Radio-immunoprecipitation of antisera.



Lane 1: MHV-infected cell lysate
Lane 2: MHV-infected cell supernatant
Lane 3: Mock-infected cell lysate
Lane 4: Mock-infected cell supernatant

BHK cells were infected with MHV-68 and radiolabelled with [^{35}S]-methionine and incubated overnight at 37°C. The cells were lysed in RIPA buffer and the lysates pre-cleared by incubating with sepharose. Lysates were incubated with i) pre-immune rabbit anti-sera or ii) rabbit anti-M4 sera and precipitated by mixing with protein A sepharose. The sepharose beads were washed, resuspended in SDS-PAGE sample buffer and electrophoresed through a 12% SDS-PAGE gel.

An attempt was made to use the anti-M4 sera as a tool to reveal the subcellular localisation of the M4 protein within MHV-68-infected BHK cells. An immunofluorescence approach (as described in section 2.5.3) was used involving a wide variety of serum concentrations. However, this approach was unsuccessful as the anti-M4 sera did not appear to bind to any of the infected cells, whereas our positive control, rabbit anti-MHV-68 polyclonal antibody, worked well.

3.1.7 M4 Protein Expression Using a Eukaryotic System

Since M4 is predicted to have a role as an immunomodulatory protein, functional studies such as the addition of M4 protein directly on cells would allow us to determine if M4 has any proliferative effects. The M4 protein expressed in the pGEX1 bacterial expression system (see figure 3.1.4) was only the first half of the protein and purification gave rise to poor yields, therefore being insufficient for functional studies. Furthermore, since the sera generated against M4 had weak activity, the use of the whole M4 protein as an antigen to produce antisera would be more useful. More success may be gained from utilising a eukaryotic mammalian expression system to express the entire M4 gene along with the benefits of eukaryotic post-transcriptional modifications.

The M4 gene was cloned into the pcDNA3.1/V5/His-TOPO plasmid using an Invitrogen TOPO TA cloning kit. The M4pc and M4B primers were used to amplify the M4 gene and the product was cloned into the vector as described in the Invitrogen manual. The pcDNA3.1/V5/His-TOPO plasmid does not direct the orientation of the gene insert, therefore the M4 gene could ligate in either orientation. The plasmid and gene were transformed into Stratagene XL-2 ultracompetent cells, resultant colonies were picked, grown overnight in liquid culture and DNA was extracted using a Qiagen miniprep kit.

Restriction digest analysis using *Bam*HI and *Hind*III were required to determine if the M4 gene had inserted in a manner so that it was reading left to right as shown in figure 3.1.7. The digests revealed that colonies either contained the M4 gene which had

inserted in the wrong orientation or that they contained an unknown DNA fragment of the same size as the M4 gene. The latter could not be explained except that a possible mutation may have occurred in either one of the two *Bam*HI sites which would prevent us recognising and detecting the M4 gene cloned in the right orientation. The *Bam*HI restriction enzyme appeared to cut the DNA once but not twice. If the mutation had occurred at the BamHI site near the 5' end of the M4 gene then the DNA would be unviable as the expression of M4 would terminate extremely early. However, if the mutation had occurred at the 3' end of the M4 gene then this would still allow us to use the plasmid for our studies as the entire M4 gene would still be expressed. Four samples containing the unknown DNA were sequenced. This revealed that the DNA was the M4 gene in the correct orientation and there were no mutations in the *Bam*HI site at the 5'end of the gene. This would allow us to assume that the mutation had occurred at the *Bam*HI site at the 3' end of the M4 and therefore would not pose a problem in our studies.

A large preparation of a sequenced pcDNA3.1/V5/His-TOPO plasmid containing the M4 gene was grown in bacteria and DNA was extracted using a Qiagen Endofree maxiprep kit. 10μg of the plasmid was transfected into 2 x 10^6 COS-7 cells (as described in section 2.4.5). The COS-7 cells were grown with selective medium until foci could be identified. After four weeks the antibiotic resistant cell foci were harvested and grown to a confluent monolayer.

The expression of M4 from the pcDNA3.1/V5/His-TOPO plasmid in the COS-7 cells was examined by making use of the hexahistidine sequence present at the 3' end of the protein. From the observation that the M4 protein contains a secretory signal, the His bind resin beads were used to purify the M4 protein from the cell supernatant (as described in section 2.3.8). Western blotting of the purified protein elutes using an anti-His antibody, the anti-M4 sera, and anti-MHV-68 polyclonal antibody did not detect an expressed protein. A large amount of non-specific protein was observed on the blots, therefore an attempt was made to remove these proteins. 0.05% Triton X-100 and

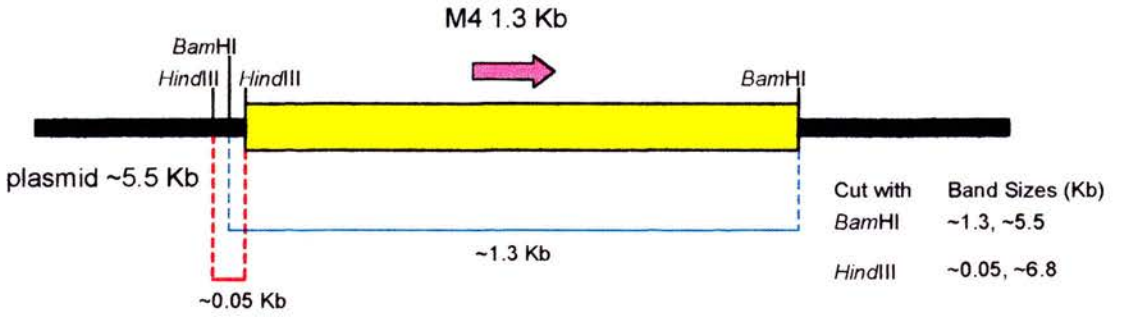
protein sepharose 4B were added to the supernatant to remove the non-specific proteins. Although these alterations did remove some of the unwanted proteins, we did not detect the expression of M4. To check if the M4 gene and the pcDNA3.1/V5/His-TOPO plasmid was still present in the COS-7 cells, DNA was extracted from the cells using a Qiagen DNeasy kit. PCR was performed on the DNA using the M4A and M4B primers within the M4 gene, and neoF and neoR primers within the neomycin resistance gene. We were unable to detect the M4 gene but were able to detect the neomycin resistance gene present in the plasmid by PCR (data not shown). Since the M4 gene appeared to have been rejected by the COS-7 cells, we decided to pursue a transient transfection approach. The pcDNA3.1/V5/His-TOPO plasmid containing the M4 gene was transfected in a similar manner into an alternative cell line, 293 cells. Cell supernatants were collected at 24 hour intervals over 72 hours post-transfection. Once again the His bind resin beads were used to purify the M4 protein from the cell supernatant (as described in section 2.3.8). However, no eluted M4 protein was visualised by western blotting.

3.1.8 Transcription of M4 During Productive Infection

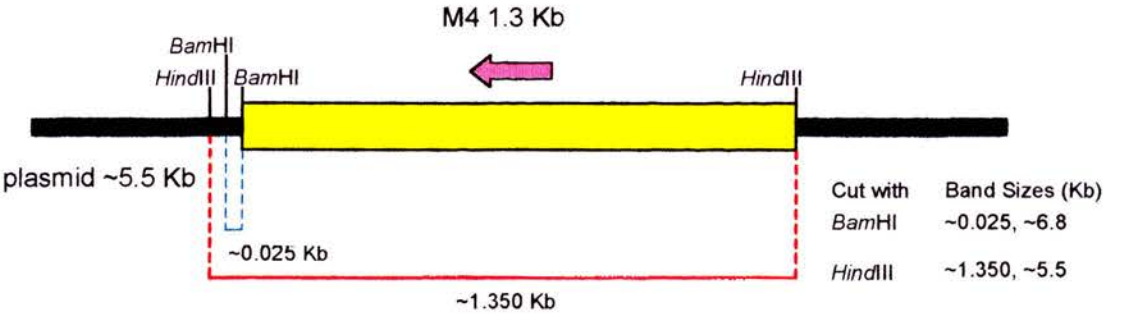
In order to further characterise the M4 gene, RNA expression studies would be beneficial by demonstrating in which phase of the MHV-68 lifecycle the M4 gene is transcribed. As previously mentioned, herpesvirus proteins are produced by genes which are categorised into one of four types; immediate early, early, late, and latency genes, depending on the time post-infection the genes are expressed. MHV-68 infected cells were harvested for immediate early, early and late RNA which was separated on a formaldehyde gel (as described in section 2.2.5). Northern blotting was performed and the RNA was hybridised with a M4 DNA probe. The developed X-ray film revealed that the M4 RNA was expressed early and late as a 1.3kb transcript during the MHV-68 lifecycle as shown in figure 3.1.8. The gp150 gene is a late gene so therefore, acting as a control, a gp150 DNA probe was used to detect the gp150 RNA transcript of 1.6kb in the lane containing the late expressed RNA. This was to confirm that the inhibitors used had worked and no leakage of RNA had occurred.

Figure 3.1.7. M4 gene orientation in the pcDNA3.1/V5/His-TOPO plasmid.

A) Correct orientation

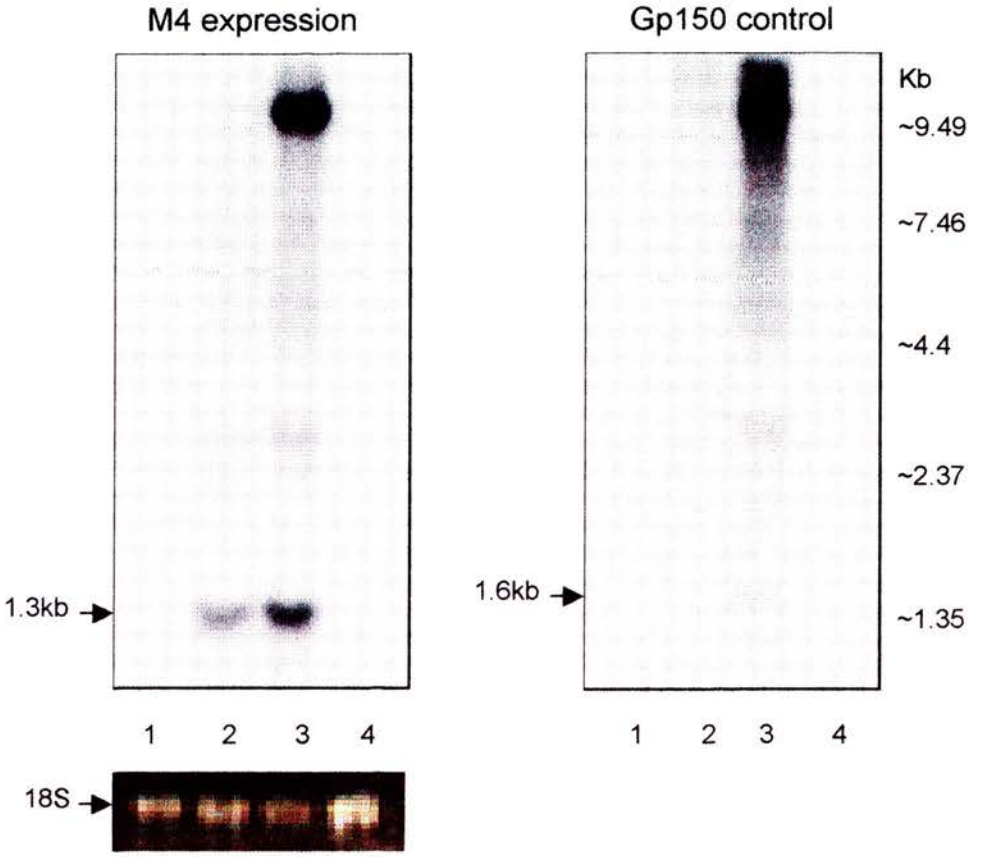


B) Incorrect orientation



M4 gene was cloned into the pcDNA3.1/V5/His-TOPO plasmid. To screen for the M4 gene cloned in the correct orientation, restriction digest analysis was performed. The *Bam*HI and *Hind*III restriction enzyme sites present in the DNA sequence of both gene and plasmid were used to determine the correct orientation. The *Bam*HI and *Hind*III restriction enzyme bands are shown by blue and red lines respectively. The M4 gene is shown by the yellow bar. The orientation of the M4 gene is shown by the pink arrow. The pcDNA3.1/V5/His-TOPO plasmid is shown by the black bar.

Figure 3.1.8 Northern analysis of M4 transcript expression.



Lane 1:	8 hr P.I. + cyclohexamide
Lane 2:	24 hr P.I. + 4'-S-Etdu
Lane 3:	24 hr P.I.
Lane 4:	0 hr P.I.

MHV-68 infected cells were harvested for immediate early, early and late RNA using the appropriate inhibitors (see section 2.4.14) and electrophoresed on a formaldehyde gel (see section 2.2.5) Northern blotting was performed and the RNA was hybridised with a M4 DNA probe. The blot was stripped and re-probed with a gp150 DNA probe. The 18S RNA subunit is shown indicating equal loading of RNA in all lanes.

3.2 Expression Discussion

Immunomodulation proteins are employed by viruses as an immune evasion strategy (Spriggs *et al.*, 1996, Alcami & Koszinowski, 2000, Lalani *et al.*, 2000, McFadden & Murphy, 2000, Tortorella *et al.*, 2000). The discovery of M4, a unique gene of MHV-68 (Virgin *et al.*, 1997) suggests that this gene is present to give an advantage to the virus and may be involved in the combating the host's defence mechanisms. Several approaches have been taken to investigate the role of the MHV-68 M4 gene including expression of the protein and transcriptional analysis.

3.2.1 Analysis of the M4 Peptide Sequence

The protein sequence of M4 was analysed using the University of Wisconsin Genetics Computer Group Sequence Analysis Software (UWGCG 9.0, Devereux *et al.*, 1984) which revealed the M4 gene had seven potential N-glycosylation sites and a heparin sulphate binding domain in the half of the peptide nearest the C-terminus. A hydrophobic signal sequence was detected at the N-terminus of the M4 gene by plotting the amino acid sequence on a hydrophobicity plot. Heparin sulphate binding domains are a common characteristic of cytokines and may be involved in the interaction between M4 protein and its receptor (Handin & Cohen, 1973, Webb *et al.*, 1993). These properties lead us to speculate that the M4 gene may be a viral-encoded cytokine. Cytokines are diverse multifunctional proteins and many viruses harbour their own cytokines (Spriggs *et al.*, 1996, Alcami & Koszinowski, 2000). A number of viral encoded secreted proteins act by interfering with host cell signal transduction pathways and innate responses. The EBV IL-10 homologue has been shown to mimic human IL-10 activities including the downregulation of IFN-γ synthesis by T cells and NK cells (Hsu et al., 1990). The M4 protein may be expressed as a secreted glycosylated protein and exert cytokine signalling by binding to its receptor via its heparin sulphate binding domain in the extracellular environment during MHV-68 infecton.

3.2.2 Expression of the M4 Protein and Generation of an Antibody

The expression of the whole M4 protein has been difficult during the project, a major obstacle being the apparent toxicity of this gene to its host cell. Initially, the expression of M4 was attempted in the pET-22b(+) plasmid, a plasmid containing a hexahistidine fusion tag. Fusion protein vectors are advantageous as they contain a tag of known size and biological function that can greatly simplify subsequent isolation, purification and detection. An advantage with the pET expression system is that the histidine tag is small and poorly immunogenic. The pET-22b(+) plasmid is under the control of the bacteriophage T7 promoter and expression is induced by providing a source of T7 RNA polymerase in the host cell, and thus the pET system is a tightly controlled system. Nevertheless, the expression of M4 was not successful in BL21(DE3) bacteria. The rationale for switching the bacterial host strains was that the BL21(DE3) strain were for general purpose expression whereas the BL21(DE3)pLysS strain was generated for high-stringency expression. The BL21(DE3)pLysS strain provides additional stability to target genes by harbouring a compatible plasmid which contains a gene which produces small amounts of T7 lysozyme. T7 lysozyme is a natural inhibitor of T7 RNA polymerase (Moffatt & Studier, 1987; Studier, 1991) by binding to small amounts of T7 RNA polymerase which have leaked before the induction procedure, thus inhibiting transcription and thereby reducing toxicity. However, the BL21(DE3)pLysS strain showed no evidence of improving M4 protein expression. Even when the M4 gene was cut in half to generate M4A and M4B, there was no evidence of protein expression. The first 500bp of the M4 gene was sequenced which revealed no mutations were present. However, the possibility of premature termination of the M4 gene cannot be ruled out as mutations may have occurred further along the sequence.

One difficulty with the pET system is that the pET-22b(+) vector does not contain a marker which is easily visible. The pGEX1 expression system has an inbuilt control in terms of being able to detect the 27kD GST protein on a SDS-PAGE gel. Furthermore, any mutation in the M4 gene would not affect expression of the GST as it is 5' to the inserted gene. However, if the gene fused to GST is toxic then this might prevent

expression of the GST.

Expression of the M4A fusion protein in the pGEX1 system was detected by a shift of the GST protein band from a molecular weight of 27kD to 47kD visualised on a Coomasie gel, and by western blotting using an anti-GST antibody. Compared to M4A, the M4B fusion protein was not successfully expressed in the pGEX1 system and this may be explained by toxicity. Furthermore, there may be motif influences in M4B not detected by amino acid analysis that inhibit expression. The M4A protein was purified using a GST sepharose column and protein elution yields were relatively low.

Another system was used to attempt to create alternative yields of the M4 protein and a stable mammalian cell line. Protein expressed in a mammalian system may be less toxic and have the same biologically activity as the native protein. The M4 gene was cloned into the pcDNA3.1/V5/His-TOPO eukaryotic expression system, a fast one step cloning strategy for the direct insertion of fresh *Taq* polymerase-amplified PCR product. This system also has the advantage that the plasmid contains a histidine tag and a V5 epitope for detection of the protein. However, expression of the plasmid containing M4 in mammalian cells was not successful following selection of G418 resistant clones. PCR analysis indicated the M4 gene had been lost while the neomycin resistance gene was retained which allowed the cells to persist under the selective medium. An explanation of the rejection may be that the M4 gene was toxic to the COS-7 cells. However, transient transfection of the plasmid into an alternative cell line, 293 cells, did not improve M4 expression. If time had permitted, the pVR1255 plasmid is an alternative vector which could have been used as it has been successfully used to express MHV-68 proteins that could not be expressed in other vectors (Atkin, 2000, Wakeling *et al*, 2001).

The M4 gene was shown to be toxic in both bacterial pET and mammalian pcDNA3 expression systems. The toxicity in bacteria may be caused by low-level readthrough transcription from the plasmid promoter (Brown & Campbell, 1993). Using an

alternative strain of bacteria that reduces toxicity did not appear to have any effect on protein expression. Expression of eukaryotic proteins can have a deleterious affect on prokaryotic cells and may cause problems in protein export machinery (Martin *et al.*, 1989). However, an attempt to express the protein in mammalian cells was not fruitful. A disadvantage of using mammalian cells as the host is that the manipulated cells can be genetically unstable which was shown in this case.

M4 protein expression is fundamental for functional characterisation studies. A major aim would be to find the receptor ligand for the M4 protein and use the M4 protein for binding studies. Alternative host expression systems include yeast cells and cultured insect cells. The advantage of the yeast system is that it lacks detectable endotoxins and only 0.5% of native proteins are exported, thereby, simplifying the isolation of secreted product. Also, large scale production of proteins is easily achieved and it is a relatively inexpensive process (Buckholz, 1993, Fleer, 1992, Sreekrishna *et al.*, 1989). However, glycosylation in yeast is not identical to mammalian systems and, thus, there may be differences in the biological activity of the protein. Cultured insect cells containing the *Baculovirus vector* have processing mechanisms similar to eukaryotic cells and have high level expression of product (Miller, 1993). However, there is a diminutive amount of information known about the glycosylation mechanisms and the product is not always fully functional. Nevertheless, a recombinant baculovirus system was successfully used to express the M3 protein (Parry *et al.*, 2000).

3.2.3 Anti-M4 Activity of Antisera

The M4A protein was used as an antigen to raise antisera in rabbits. The antisera were tested against western blots of the M4A protein expressed in the pGEX system. The antiserum bound to the M4A fusion protein but did not detect any protein by western blot in MHV-68 infected lysates. Since the binding to the fusion protein could be due to M4 and not GST recognition, we decided to pursue the more sensitive radioimmunoprecipitation approach. In radiolabelled MHV-68-infected cell lysates, the antisera bound to a protein of 44kD, the predicted size of M4, and an unknown protein

band size of ~80kD. Furthermore, the antisera precipitated an unknown protein of ~60kD in radiolabelled infected cell supernatant which had a "fuzzy" appearance indicative of a processed protein with variable molecular weight. Several different protein complexes may form between M4 and unknown protein molecules within the cell as well as being secreted into the supernatant. Further experiments are required to reveal the nature of these unknown proteins.

The subcellular localisation of the M4 protein within MHV-68-infected BHK cells was investigated using the antiserum generated against M4. Immunofluorescence studies revealed that the antiserum did not detect the presence of M4 in the infected cells. Interestingly, the M4 sequence harbours a secretory signal and the immunoprecipitation studies have demonstrated that a certain proportion of M4 is secreted. Therefore, it is possible that a large amount of the M4 protein may be secreted and therefore not enough protein is retained within the infected cell to be detected by the antisera. Further studies using inhibitors such as tunicamycin which blocks steps in glycosylation and thus block secretion of proteins could be used as an alternative strategy (Elbein, 1984).

Although the generation of an antiserum against M4 has been successful, the fact that it did not bind M4 protein on western blots or detect antigen in infected cells suggested that it was a weak antiserum and without further purification, the antibody was not useful for further studies. The presence of the GST protein attached to the M4A protein may have resulted in a stronger antibody being raised against the GST rather than the M4A protein. In retrospect, the GST protein could have have been removed before using the fusion protein as an antigen. Also, it may have been possible to remove the anti-GST antibodies from the rabbit serum using a purification column containing the GST and then add the serum to another column containing the M4 fusion protein to bind and thus purify the anti-M4 antibodies. However, time did not permit us to grow and purify large quantities of GST and M4 fusion protein to test this strategy.

3.2.4 Transcription of M4 During Productive Infection

Northern analysis was used to examine the *in vitro* transcription of M4 during productive infection. M4 RNA transcripts were expressed early and late as a 1.3kb transcript during the MHV-68 lifecycle. To distinguish immediate early and early RNA transcribed post-infection, the inhibitory compounds cyclohexamide and 2'deoxy-5'ethylβ-4'thiouridine (4'-S-EtdU) (Rahim *et al.*, 1996) were used respectively. Cycloheximide suppresses viral protein synthesis and 4'-S-EtdU blocks viral DNA replication. Early genes require the translation of immediate early transcripts for expression while late transcripts require viral DNA synthesis for transcription (Roizman & Sears, 1996). Another MHV-68 early-late gene detected by northern analysis is the M3 gene which has been shown to be a functional chemokine binding protein (van Berkel *et al.*, 1999, Parry *et al.*, 2000, van Berkel *et al.*, 2000). The finding that M4 is expressed early is consistent with it having an immunomodulatory role. If time had permitted, it would be have been interesting to characterise the mRNA structure of M4. Rapid amplification of cDNA ends (RACE) and S1 nuclease protection could have been used to map the 5' and 3' ends of the M4 transcript as was performed with the characterisation of the M3 gene (van Berkel *et al.*, 1999).

Chapter Four:

Recombinant Virus Studies

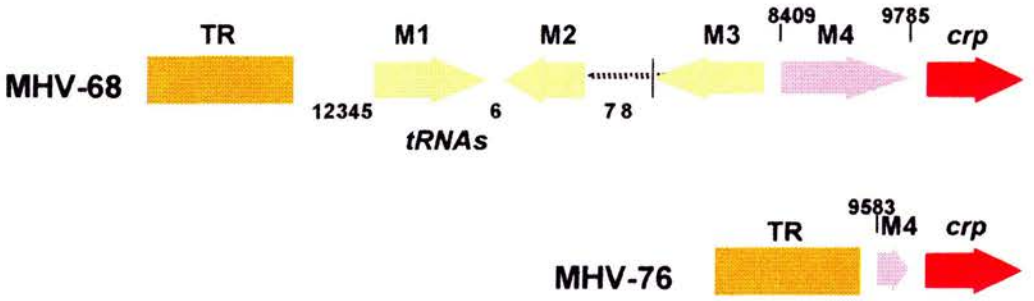
4.1 Recombinant Virus Results

The study of mutant and recombinant viruses in which specific genes have been mutated, deleted or inserted are fundamental in elucidating the function of viral genes. Herpesviruses harbouring mutated genes have been discovered and isolated on the basis of altered plaque phenotype (Ejercito *et al.*, 1968). The invaluable use of recombinant DNA technology has now permitted the generation of specific recombinant viruses.

The molecular biology studies of gammaherpesviruses have had major set backs due to the inability of these viruses to grow permissively in cell cultures. MHV-68 does not encounter this problem, being replication efficient in cell culture. This property allows us to take advantage of MHV-68 and utilise it for genetic manipulation studies of viral genes. The M genes have an unknown function. However, with the crucial assistance of recombinant virus technology, possible functions have been attributed to some of these genes (Simas *et al.*, 1998, Clambey *et al.*, 2000). MHV-68 recombinant viruses have been extremely useful in our understanding of the virus-host interactions which occur with this infection (Clambey *et al.*, 2000; Hoge *et al.*, 2000; Simas *et al.*, 1998; van Dyk *et al.*, 2000).

A recombinant virus only lacking a functional M4 gene has not been generated. In order to determine the function of M4 we had two strategies. Our first strategy was to construct an MHV-68 virus which lacked the entire M4 gene, a M4 "knock out" (KO) virus. It has been shown previously with gammaherpesviruses including MHV-68, that these viruses do not tolerate recombination events very well (Simas *et al.*, 1998). Therefore, if our first strategy failed we had an alternative plan with the use of MHV-76 as a tool. As previously described (see section 1.5.6), MHV-76 appears to be a deletion mutant of MHV-68 which lacks the first 1-9538bp as shown in figure 4.1 (Macrae *et al.*, 2001). Thus, it does not encode M1, M2, M3, the viral tRNAs and crucially for our purposes, M4. An opportunity was present to generate MHV-76 which contained the entire M4 gene, a M4 "knock in" (KI) virus. Generation of either of these recombinant viruses would be a critical tool for our functional studies. Essentially our aim would be

Figure 4.1. Comparison of MHV-68 and MHV-76 left hand regions.



Left hand regions of the MHV-68 and MHV-76 genome are shown in the diagram. The MHV-76 genome lacks the first 1-9583bp of the MHV-68 genome (Macrae *et al.*, 2001). The M4 gene is shown by the pink arrow. Terminal repeats (TR) are shown by orange bars. M1-M3 genes are shown by yellow arrows. The complement regulatory protein (crp) is shown by the red arrow. The 8 viral tRNAs are numbered 1-8.

to explore the pathogenesis of the recombinant virus *in vivo*. Mice would be infected with the purified recombinant virus and analysed by titration of infective virus in the lung and reactivation of latent virus in the spleen and mediastinal lymph nodes (MLN) in comparison with the wild type virus.

4.1.1 M4 Knock-Out Recombinant (M4KO) Virus Cloning Strategy

The cloning strategy used to generate a M4 knock out recombinant (M4KO) virus is outlined in figure 4.1.1. Our aim was to delete the M4 gene from the MHV-68 genome and insert in its place a cassette encoding the green fluorescent protein (GFP) via homologous recombination. The GFP gene was originally derived from the jelly fish *Aequoria victoria* (Zolotukhin *et al.*, 1996) and is now a widely used marker due to its ability to emit a green fluorescence upon excitation by UV light of maximum 488nm wavelength. The green fluorescence is expressed during productive viral replication as a result of the gene being under the control of the constitutive CMV immediate early (CMVIE) promoter. Due to the ease of detection of the GFP gene, this tool has been widely used in the construction of recombinant herpesviruses (Prichard 1999, Duboise 1996).

A three-step cloning strategy as depicted in figure 4.1.1 was used to generate the M4 recombinant construct. Flanking regions of DNA directly after each end of the M4 gene were amplified by PCR. The M41F and M41R primers were used to amplify a flanking region to the left of M4 and the M42F and M42R(2) primers were used to amplify a flanking region to the right of M4. The flanking regions were ~600bp in length to allow for homologous recombination. The PCR products were connected together using complementary base pairs to form a single PCR product which flanked both sides of M4 using the M41F and M42R(2) primers. The M4 flanking DNA PCR product was purified and cut with *Bam*HI and *Xho*I and ligated into corresponding sites in the pBluescript II KS^+ (pKS) vector. The ligation mix was used to transform competent *E.coli* and resultant colonies were picked and grown up in liquid culture. The plasmid DNA was extracted and analysed for the correct construct by restriction digestion

analysis. The correct plasmid construct containing the M4 flanking regions was digested with *Acc*I. A construct (kindly provided by Dr. D. J. Roy) containing the GFP gene flanked by the CMVIE promoter and the Simian virus 40 polyadenylation signal (SV40pA), was amplified from the plasmid pEGFP-C1 using primers CMV1 and SV40. The pEGFP-C1 multiple cloning site, which is situated at the 3' end of the GFP coding region, was excised prior to amplification, which assisted subsequent cloning of the GFP gene. The plasmid was digested with AccI and ligated into the corresponding AccI sites within the M4 flanking DNA in the pKS^+ vector and transformed into competent *E. coli*, grown up and analysed as described previously. Restriction digest analysis was used to determine the GFP cassette was in the same transcriptional orientation as the M4 flanking DNA. The M4KO construct consisted of the pKS^+ vector containing the GFP gene flanked with DNA which flanked the left and right hand of the M4 sequence. A large scale preparation of the M4KO plasmid was made using a Qiagen endofree maxi-prep kit. The M4KO plasmid was digested with *Bam*HI and *Xho*I to remove the M4KO cassette from the pKS^+ vector to avoid a recombination event which might lead to the entire plasmid being incorporated into the MHV-68 genome.

BHK cells were co-transfected with 15μg of MHV-68 DNA and 10μg of the excised M4KO cassette. The cells were overlaid with agar 24 hours post-transfection. At four days post-transfection, wild type virus plaques were observed using the light microscope and green fluorescent plaques were visible using a UV microscope.

4.1.2 Purification of M4KO Virus

Plaque purification was used to isolate wild type MHV-68 plaques from the recombinant plaques (see section 2.4.13). Green plaques were picked with a Pasteur pipette and serially diluted and plated with a cell line, C127, which forms a more easily detectable plaque in comparison to BHK cells. Six rounds of plaque purification were performed which did not appear to increase the purification of the green virus. The C127 cells were replaced with αβSV1 cells. This is a murine embryo fibroblast cell line which lacks the type I interferon receptor (kindly provided by Dr. J. P. Stewart). Previous studies have

shown that MHV-68 plaques more efficiently in this cell line than in BHK or C127 cells (A. Macrae, personal communications).

4.1.3 PCR of M4KO Virus

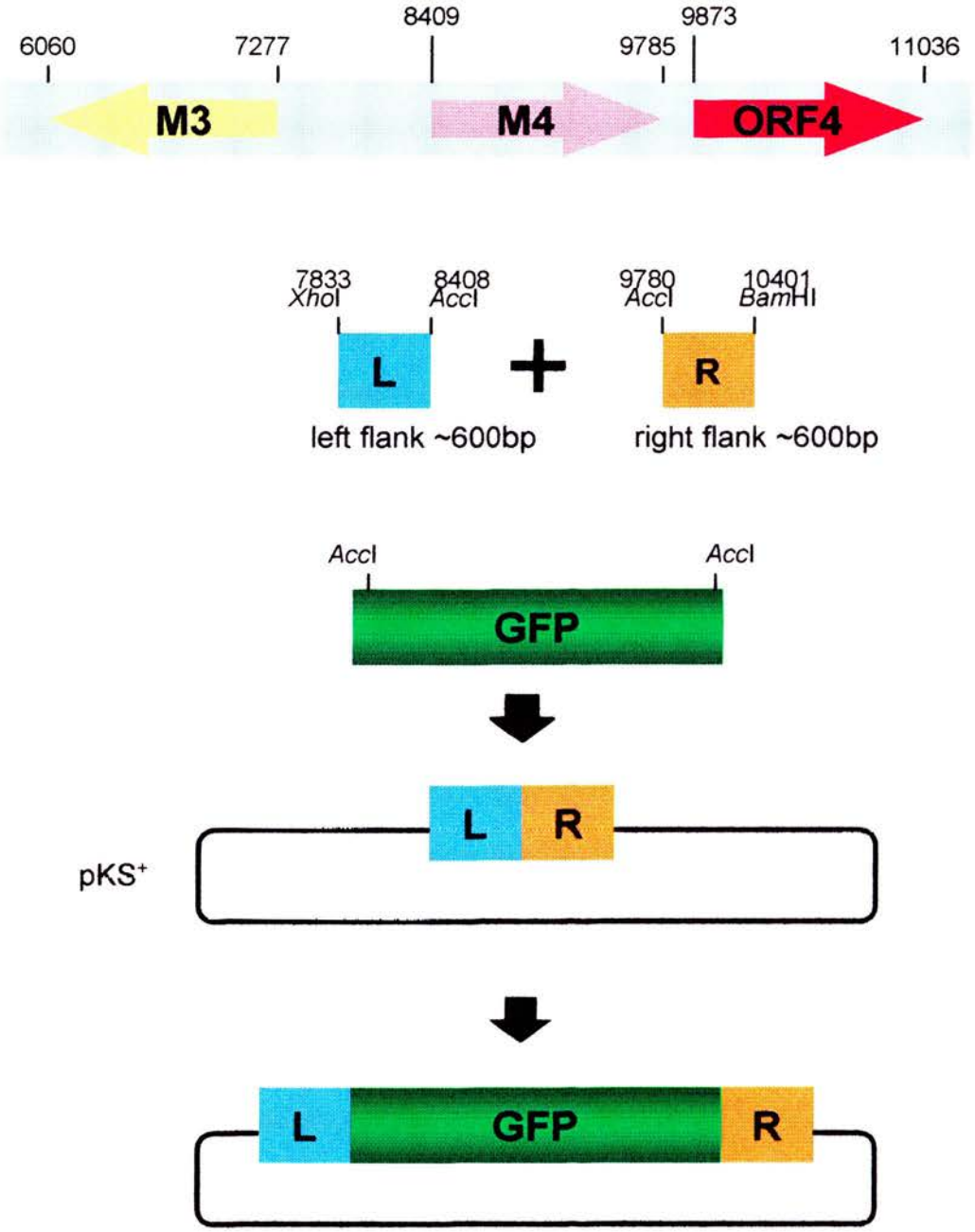
Virus DNA was extracted from cells infected with a single isolated green plaque and analysed by PCR. The positions of the primers used are in figure 4.1.3. Primers M4LH1 and GFP2 were used to detect whether the recombinant construct had recombined in the correct region of genome, GFP1 and GFP 2 were used to detect the presence of the GFP gene, and M4LH1 and M4Arev were used to detect the presence of M4. The PCR failed to demonstrate homologous recombination between the MHV-68 genome and the M4 knock out recombination construct had occurred in the desired manner as seen in figure 4.1.1. The M4LH1 and M4Arev primers amplified the 1.3kb M4 gene, thereby demonstrating the presence of M4. GFP1 and GFP2 primers revealed that the 1.6kb GFP gene had recombined into the MHV-68 genome. However, PCR with M4LH1 and GFP2 primers demonstrated the GFP gene recombination had not occurred in the correct region and had not disrupted and removed the M4 gene.

Time limitations did not permit us to perform a Southern analysis with the recombinant DNA which would have revealed the exact location of the non-homologous recombination.

4.1.4 M4 Knock In Recombinant (M4KI) Virus Cloning Strategy

Since our attempt to generate MHV-68 without the M4 gene had been futile, we adopted our alternative plan to utilise MHV-76 by generating MHV-76 with the M4 gene inserted. The three step cloning strategy is shown in figure 4.1.4a. A 1.2kb *Pst*I fragment of the MHV-68 terminal repeat had previously been cloned into a pUC13 plasmid (Efstathiou *et al.*, 1990b). Restriction analysis revealed that the terminal repeat was opposite to our desired orientation. We attempted to switch the orientation of the terminal repeat by standard cloning methods but the fragment appeared to harbour preferential orientation and we were unsuccessful. Therefore, we cut the terminal repeat

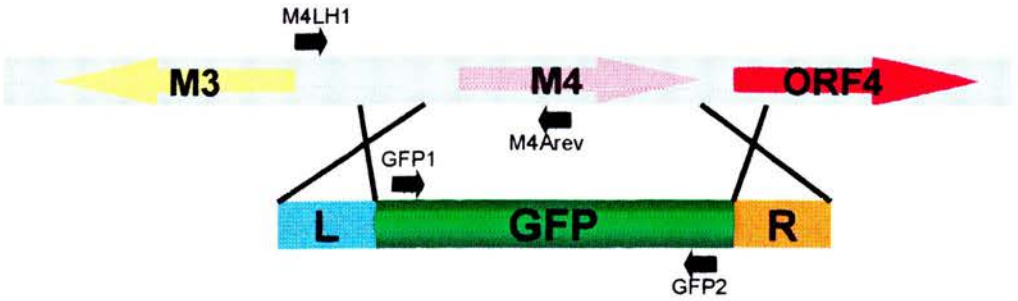
Figure 4.1.1 Cloning strategy of M4KO construct.



The M4 gene left (L) and right (R) flanking regions were amplified via PCR using *Taq* polymerase. The L and R products were joined via PCR to produce one DNA fragment and cloned into the *Bam*HI and *Xho*I restriction sites of the pBluescript II KS$^+$ pKS$^+$ vector. The GFP gene was inserted between the left and right flanking DNA using the *Acc*I restriction site. L - left flanking DNA of M4, R - right flanking DNA of M4, GFP - green fluorescent protein.

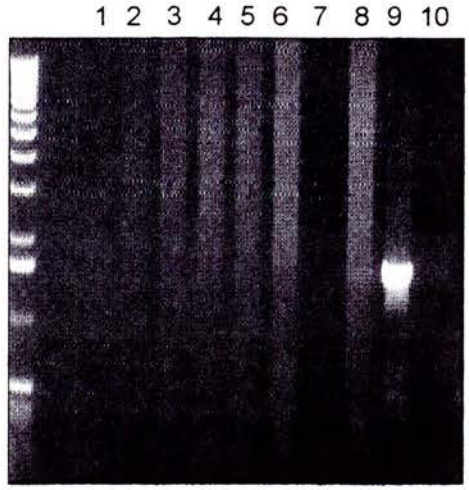
Figure 4.1.3 PCR of M4KO virus.

i)



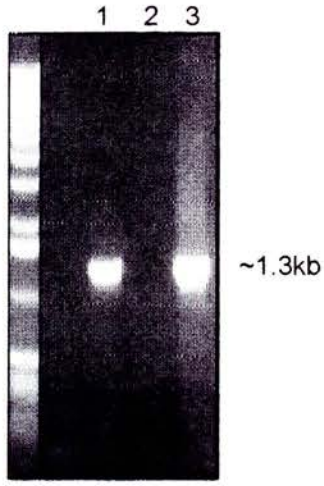
ii)

Lane
1. 1µl KO DNA + M4LH1/GFP2
2. 2µl KO DNA + M4LH1/GFP2
3. 3µl KO DNA + M4LH1/GFP2
4. 4µl KO DNA + M4LH1/GFP2
5. 6µl KO DNA + M4LH1/GFP2
6. 10µl KO DNA + M4LH1/GFP2
7. No DNA + M4LH1/GFP2
8. 10µl KO DNA + GFP1/GFP2
9. 4µl KO DNA + GFP1/GFP2
10. no DNA + GFP1/GFP2



iii)

Lane
1. MHV68 DNA + M4LH1/M4Arev
2. No DNA + M4LH1/M4Arev
3. KO DNA + M4LH1/M4Arev



i) Diagram of homologous recombination event between the M4 knock-out recombinant construct and the MHV-68 genome. Green knock-out recombinant virus plaques were picked and propagated in BHK cells. DNA was extracted from one sample as described in section 2.1.19. PCR was performed on a range of dilutions of DNA using ii) M4LH1, GFP1 and GFP2 primers and iii) M4LH1 and M4Arev primers and *Taq* polymerase. Primer positions are shown in i) as black arrows. L - left DNA flank, R - right DNA flank, GFP - green fluorescent protein, KO - knock-out virus.

from pUC13 using *Pst*I and re-cloned the fragment into a pUC18 plasmid. In contrast to pUC13, our desired orientation for the terminal repeat fragment in pUC18 was successfully achieved The correct orientation in pUC18 was demonstrated by restriction analysis. Our next step was to amplify by PCR using primers MHV76A and MHV76B, a 2.6kb fragment from the MHV-68 genome containing the M4 gene and flanking DNA. The MHV76A and MHV76B primers were designed to harbour *Bam*HI and *Eco*RI restriction sites respectively. The two restriction enzymes were used to cut the purified PCR product and the pUC18 plasmid. The M4 gene and flanking DNA were cloned into the pUC18 plasmid containing the terminal repeat. The same GFP gene used for the M4 knock out virus (see section 4.1.1) was amplified as previously described, cut with *Bam*HI and *Bgl*II and cloned into the corresponding site in the pUC18 plasmid. Restriction digest analysis was used to determine that the GFP cassette was in the same transcriptional orientation as the terminal repeat and the M4 gene. A large scale preparation of the M4 knock in (KI) plasmid was made using a Qiagen endofree maxi-prep kit. The M4KI plasmid was digested with *Hind*III and *Eco*RI to remove the M4KI cassette from the pUC18 plasmid.

BHK cells were co-transfected with 10μg of MHV-76 DNA and 10μg of the excised M4KI cassette. The cells were overlaid with agar 24 hours post-transfection. At four days post-transfection, wild type virus plaques were observed using the light microscope and green fluorescent plaques were visible using a UV microscope (figure 4.1.4b).

4.1.5 Purification of M4KI Virus

As previously described with the purification of the M4KO virus, plaque purification was used to isolate wild type MHV-76 plaques from the M4KI recombinant plaques (see section 2.4.13). Green plaques were picked with a Pasteur pipette and serially diluted and plated with BHK cells. However, in contrast to our method with the M4KO virus, only one round of plaque purification was performed before we analysed the recombinant virus by PCR.

4.1.6 PCR of M4KI Virus

Virus DNA was extracted from cells infected with a single isolated green plaque using a Qiagen DNeasy kit and analysed by PCR. The positions of the primers used are in figure 4.1.6. Primers M4A and M4GFP76 were used to detect whether the recombinant construct had recombined in the correct region of genome, and M4A and M4B were used to detect the presence of M4. The PCR demonstrated homologous recombination between the MHV-76 genome and the M4 knock in recombination construct had occurred in the desired manner as seen in figure 4.1.6. The M4A and M4GFP76 primers amplified the expected 2.8kb fragment indicating that homologous recombination had been successful. The M4A and M4B primers amplified the 1.3kb M4 gene, thereby demonstrating the insertion of M4.

4.1.7 Southern Analysis of M4KI Virus

Now we had evidence that the generation of the M4KI virus had been successful, we performed another two rounds of plaque purification. At this point, the recombinant virus had been purified to the standard that no wild type plaques were visible under the light microscope. PCR does not detect the purity of the recombinant virus, however, a Southern analysis would inform us if wild type virus was present. MHV-68, MHV-76, and the M4KI virus were propagated on BHK cells and their DNA was extracted as described in section 2.1.19. 1μg/lane of each viral DNA digested with either BamHI, BglII or EcoRI, were electrophoresed on two 0.7% TAE gels alongside 5μg of 1kb DNA molecular weight markers. The gel was blotted overnight by capillary transfer to nylon membranes as described in section 2.1.22 and fixed by UV cross-linking. Each blot was hybridised with a different ^{32}P-labelled DNA probe corresponding to either (a) the first 590bp of M4 or (b) HindIII G fragment of the MHV-68 genome. The restriction enzyme maps of the left hand end of MHV-68, MHV-76 and the M4KI virus and expected band sizes with the two different probes are detailed in figure 4.1.7a. The hybridised southern blots are shown in figure 4.1.7b and the band sizes that distinguish the three different viruses are confirmed. The blot hybridised with the M4 probe confirmed again that the M4 gene is only present in MHV-68 and the M4KI virus and

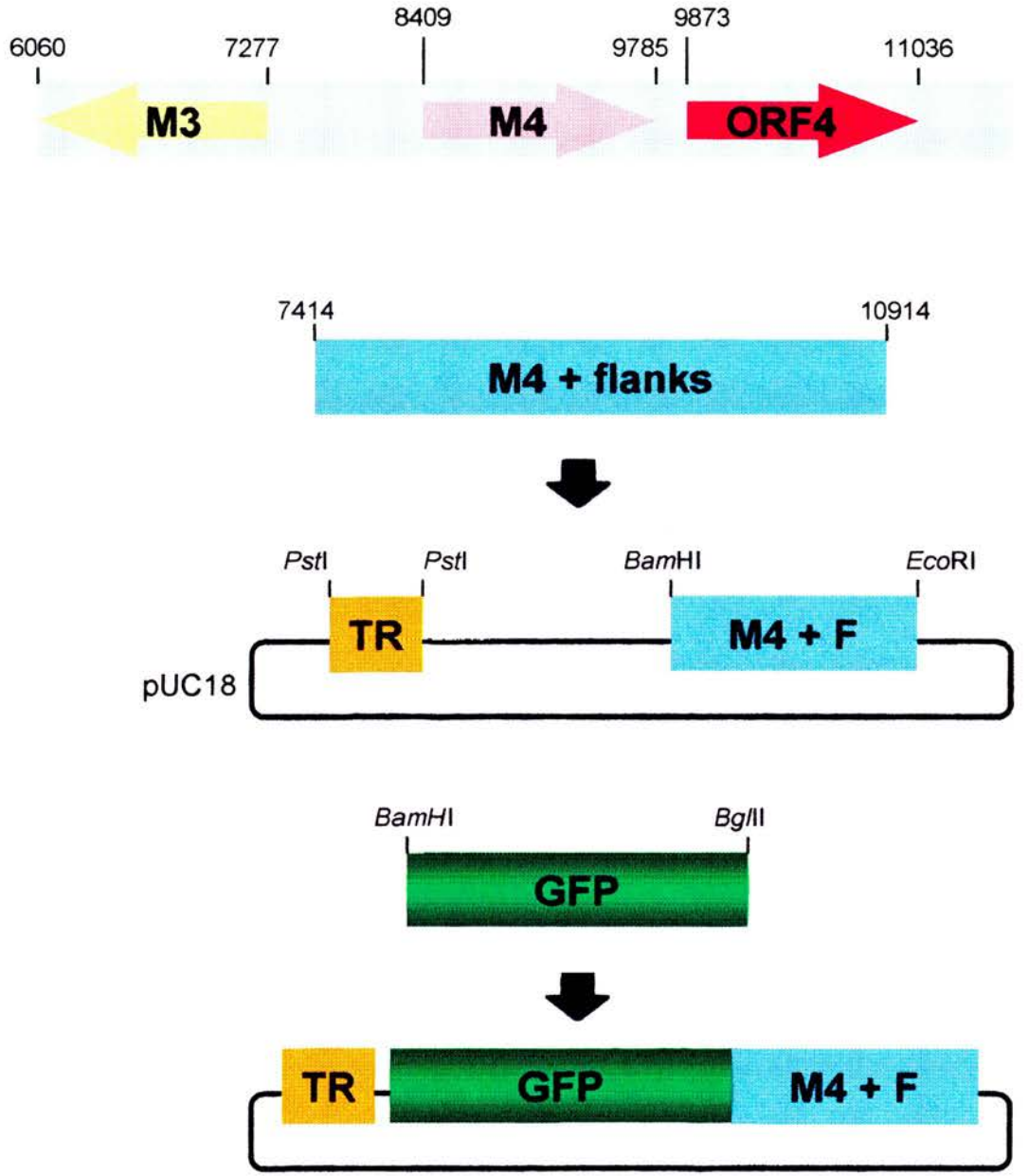
the generation of the M4KI virus had been successful. Hybridisation with the HindIIIG probe revealed that the M4KI virus stock was pure due to the inability to detect wild type MHV-76. It was apparent from the blots that the probes detected a series of ladder-like DNA fragments which may alter the sizes of some of the bands. These fragments have previously been detected and is a result of the viral genome harbouring variable copies of 1.2kb terminal repeats (Efstathiou *et al.*, 1990).

4.1.8 Transcript Expression of M4KI Virus

Before we used the M4KI virus for in vivo studies, it was essential to establish M4 transcript expression. Whole RNA was extracted using a RNeasy kit (Qiagen) from BHK cell monolayers infected overnight with either MHV-68, MHV-76 or M4KI recombinant virus. The RNA preparations was DNaseI treated (as described in section 2.2.3) to remove any viral or cellular DNA. Complementary DNA (cDNA) was reverse transcribed from RNA (as described in section 2.2.4) and used for PCR with the M4A and M4Arev primers and β-actin as seen in figure 4.1.8a. The M4A and M4Arev primers amplified M4 DNA present in the MHV-68 and M4KI cDNA but not in the non-infected or MHV-76 cDNA. DNaseI treated RNA was also used for PCR to establish that the M4 DNA detected was not present in the samples before cDNA preparation. The β-actin primers were used as a housekeeping control to show that RNA was present in all samples.

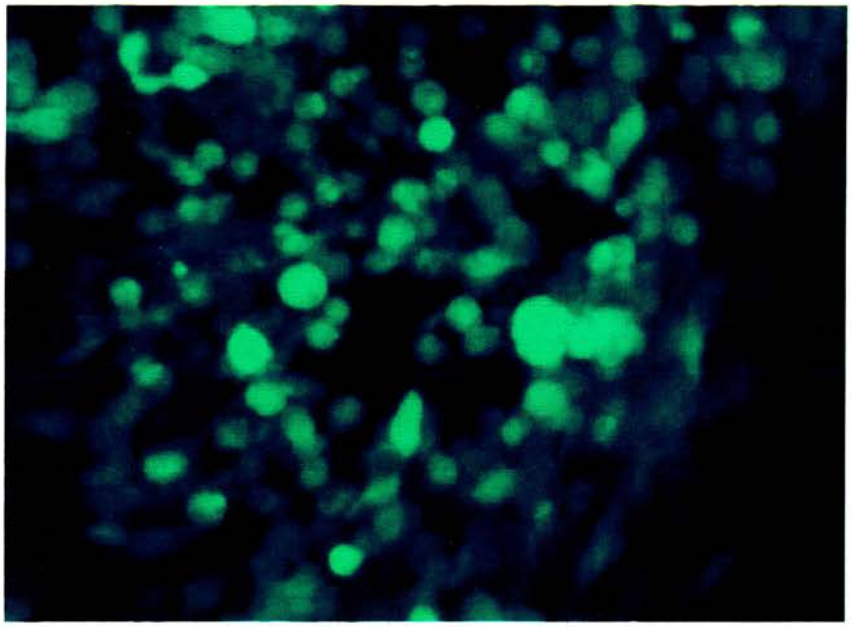
In addition, northern blotting was used to analyse M4 RNA expression. The DNaseI treated RNA from the previous experiment was electrophoresed on a formaldehyde gel, blotted onto a nylon membrane and hybridised with a M4 DNA probe (as described in section 2.2.6). The northern analysis confirmed the RT-PCR results by showing the M4 DNA probe had bound the M4 transcript of 1.3kb in the MHV-68 and M4KI RNA but not the MHV-76 RNA (figure 4.1.8b).

Figure 4.1.4a Cloning strategy of M4KI construct.



The M4 gene and flanking regions were amplified via PCR using *Taq* polymerase. The M4 PCR product was cloned into the *Bam*HI and *Eco*RI restriction sites of the pUC18 plasmid containing the terminal repeat (TR). The GFP gene was inserted between the TR and M4 gene using *Bam*HI and *Bgl*II restriction sites. TR -terminal repeat, F - flanking regions of M4, GFP - green fluorescent protein.

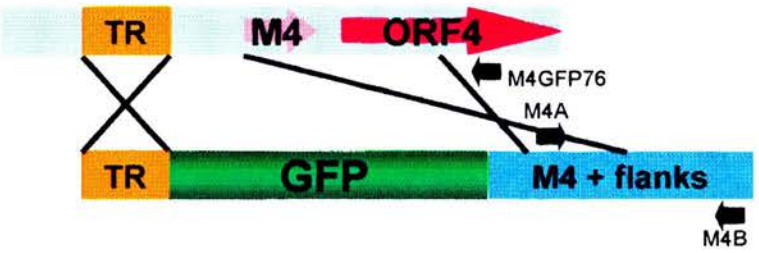
Figure 4.1.4b M4KI virus plaque.



BHK cells were co-transfected with MHV-76 DNA and M4KI cassette. Cells were overlaid with agar 24 hours post-transfection. At four days post-transfection, green fluorescent plaques were visible using a UV microscope.

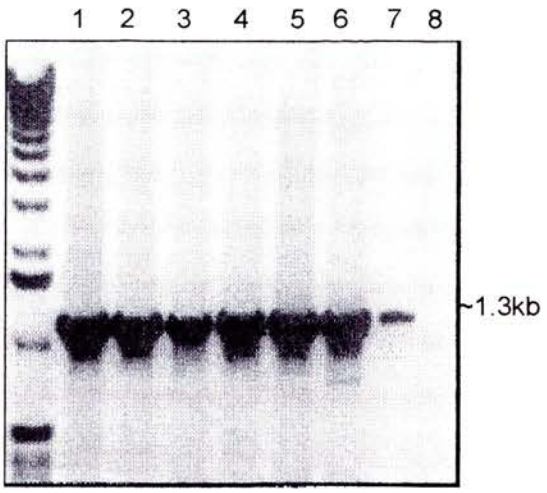
Figure 4.1.6 PCR of M4KI virus

i)



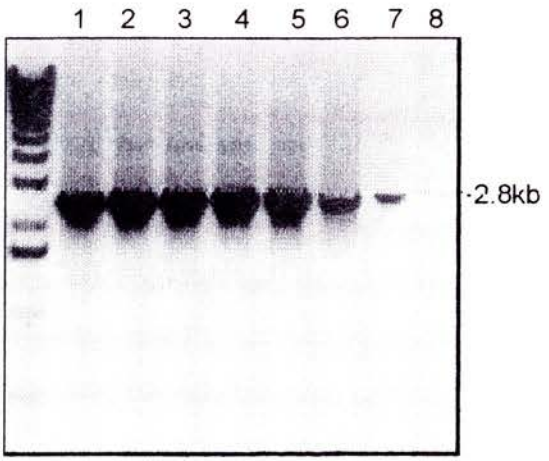
ii)

Lane
1. KI4 virus DNA + M4A/M4B
2. KI5 virus DNA + M4A/M4B
3. KI6 virus DNA + M4A/M4B
4. KI7 virus DNA + M4A/M4B
5. KI8 virus DNA + M4A/M4B
6. KI9 virus DNA + M4A/M4B
7. MHV-68 DNA + M4A/M4B
8. No DNA + M4A/M4B



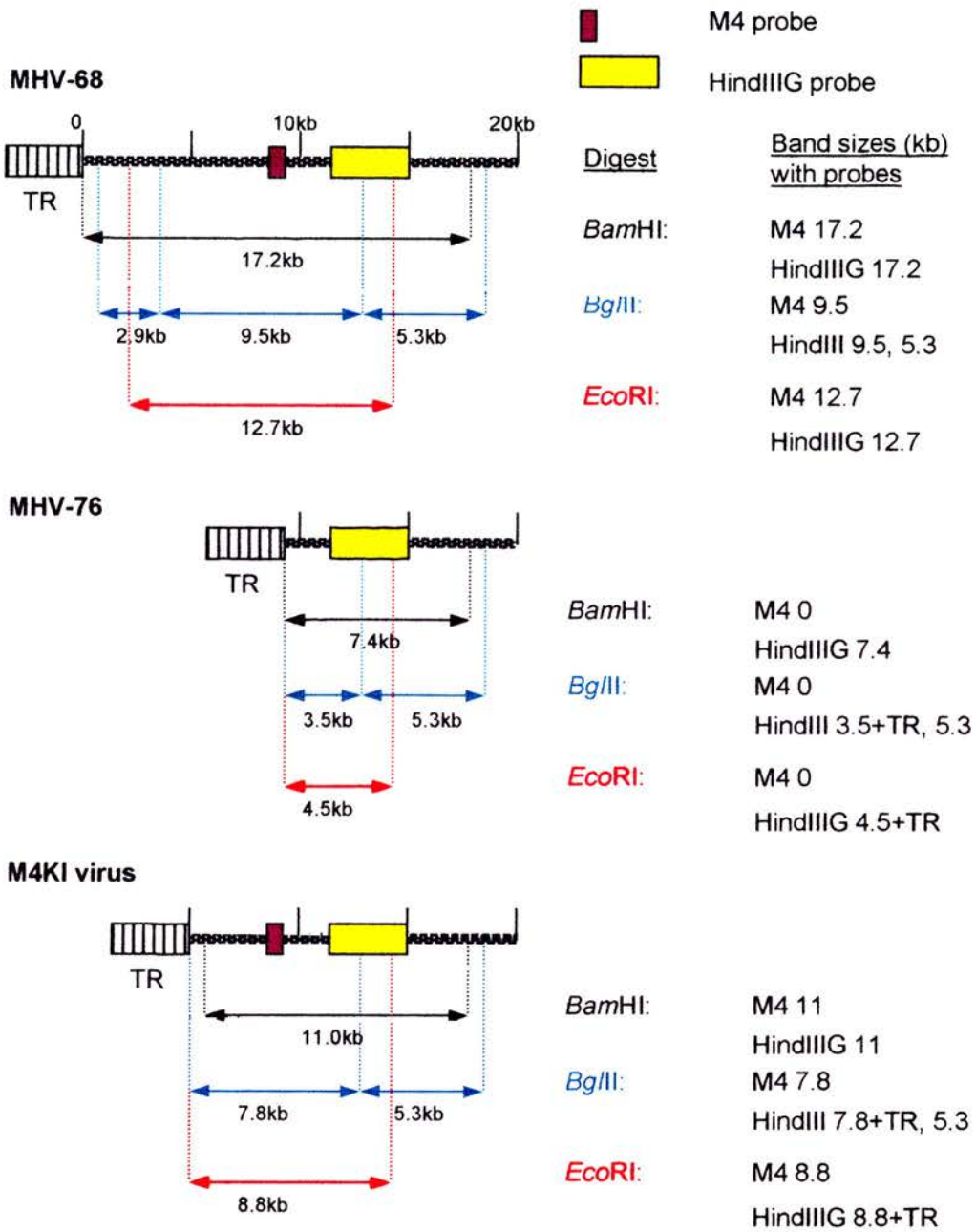
iii)

Lane
1. KI4 virus DNA + M4A/M4GFP76
2. KI5 virus DNA + M4A/M4GFP76
3. KI6 virus DNA + M4A/M4GFP76
4. KI7 virus DNA + M4A/M4GFP76
5. KI8 virus DNA + M4A/M4GFP76
6. KI9 virus DNA + M4A/M4GFP76
7. MHV-68 DNA + M4A/M4GFP76
8. no DNA + M4A/M4GFP76



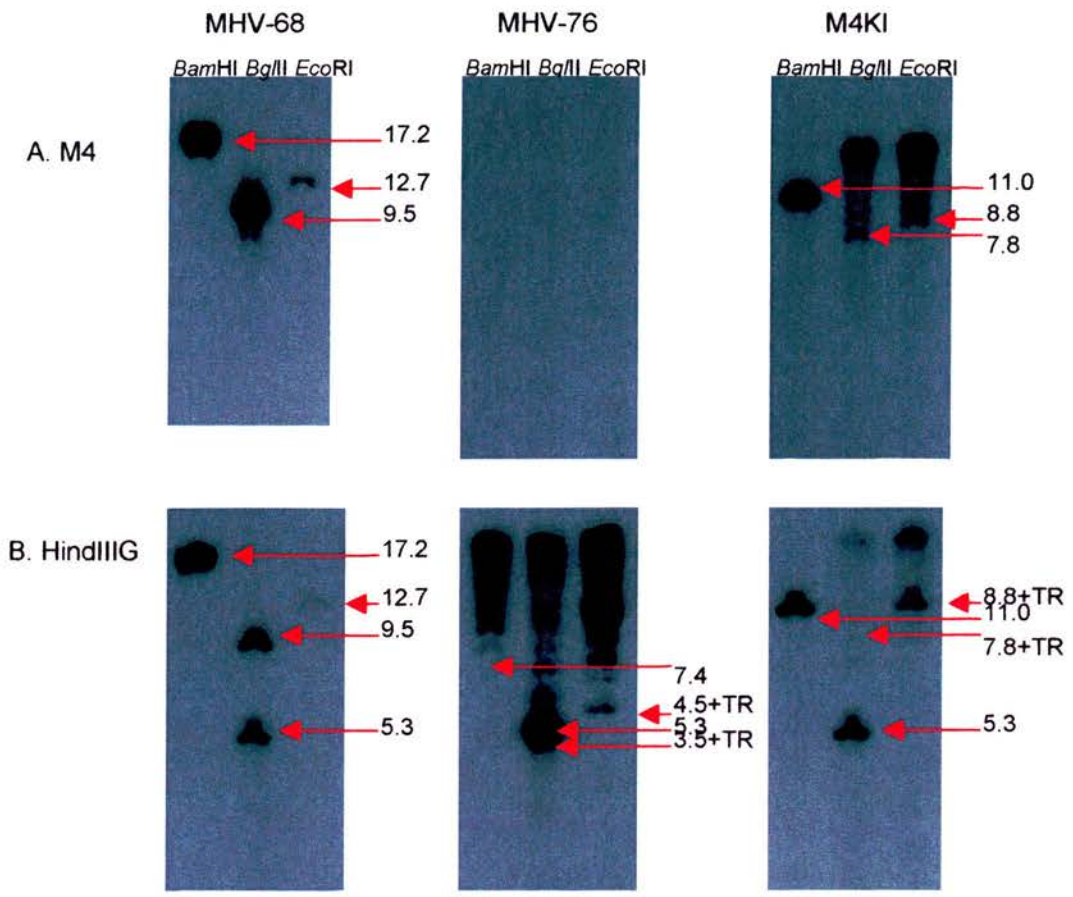
i) Diagram of homologous recombination event between the M4KI construct and the MHV-76 genome. Six green knock-in recombinant virus plaques (KI4-KI9) were picked and propagated in BHK cells. DNA was extracted using a Qiagen DNeasy kit. PCR was performed using ii) M4A and M4B primers and iii) M4A and M4AGFP76 primers and *Taq* polymerase. Primer positions are shown in i) as black arrows. TR - terminal repeats, GFP - green fluorescent protein, KI - knock-in virus.

Figure 4.1.7a Restriction maps of MHV-68, MHV-76 and M4KI virus.



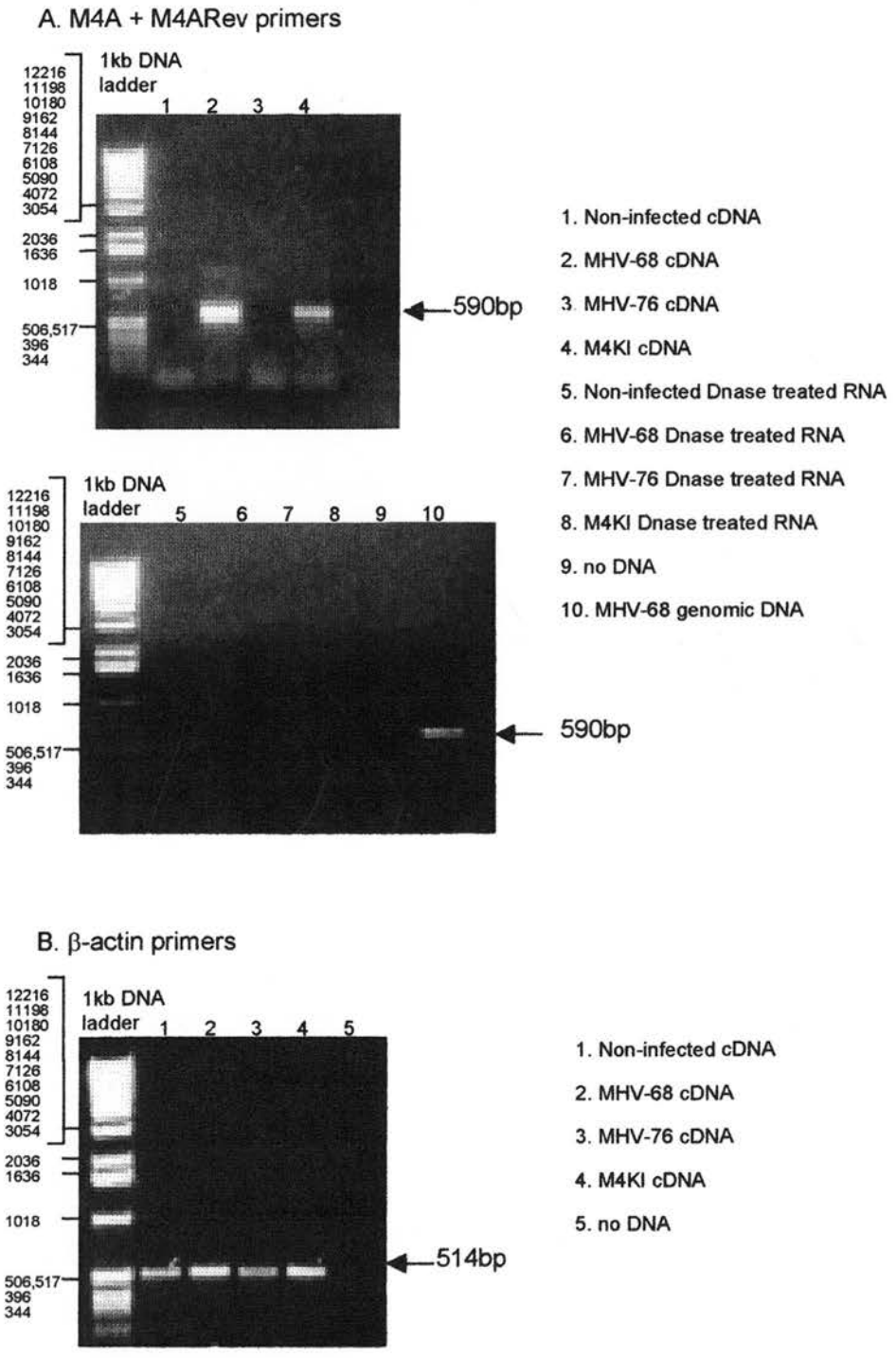
Restriction maps of the left hand region of the MHV-68, 76 and MKI virus. The predicted band sizes are shown for restriction digests of wild type virus and recombinant virus DNA digested with one of three enzymes, *Bam*HI, *Bgl*II or *Eco*RI. Band sizes are detected using probes for the M4 gene and the HindIIIG fragment. TR - terminal repeat, KI - knock-in.

Figure 4.1.7b Southern analysis of MHV-68, MHV-76 and M4KI virus.



MHV-68, MHV-76 and M4KI recombinant virus were propagated on BHK cells and DNA was extracted as described in section 2.1.19. 5μg of each viral DNA were digested overnight with either *Bam*HI, *Bgl*II or *Eco*RI. 1μg/lane of viral DNA were electrophoresed on two 0.7% TAE gels alongside 5μg of 1kb DNA molecular weight markers. The gels were blotted overnight by capillary transfer to nylon membranes as described in section 2.1.22 and fixed by UV cross-linking. Each blot was hybridised with a different ^{32}P-labelled DNA probe corresponding to either (a) the first 590bp of M4 or (b) HindIII G fragment of the MHV-68 genome. TR - terminal repeat, KI - knock-in virus.

Figure 4.1.8a Reverse transcription PCR of M4KI virus.



Reverse transcription-PCR (RT-PCR) was performed using *Taq* polymerase with A) M4A and M4ARev primers, and B) β-actin primers. Products are electrophoresed on a 0.8% agarose gel. KI - knock in, cDNA - complementary DNA

Figure 4.1.8b RNA expression of M4KI virus.

Lane 1: mock-infected RNA

Lane 2: MHV-68 RNA

Lane 3: MHV-76 RNA

Lane 4: M4KI RNA

BHK cells were infected with either MHV-68, MHV-76 or M4KI virus overnight. RNA was extracted using RNAzolB (Sigma) and 5μg/lane of RNA was electrophoresed on a formaldehyde gel overnight. The gel was blotted with nylon membrane and hybridised with the M4 gene.

4.1.9 Growth of M4KI Virus

The growth characteristics *in vitro* of the M4KI virus were established using the one step growth curve method (as described in section 2.4.20). MHV-68, MHV-76 and the M4KI virus stocks were titrated simultaneously on the same BHK-21 cells prior to the experiment to provide a reliable comparison between the three viruses. 2 x 10^5 BHK cells were infected per timepoint at 5 pfu/cell. Cells were harvested at 0, 4, 8, 12, 20, 24, 30, 36, 48, 60 and 72 hour post-infection and titrated on BHK-21 cells (as described in section 2.4.18). The one step growth curves of MHV-68, MHV-76 and the M4KI virus are shown in figure 4.1.9. As reported previously, MHV-68 replicated efficiently and attained maximal titres at around 60 hours post-infection and the growth kinetics of MHV-76 was similar to MHV-68 (Sunil-Chandra, 1991, Macrae *et al.*, 2001). The growth curves demonstrated there is no difference in the growth of the M4 KI virus *in vitro* on BHK-21 cells compared to MHV-68 and MHV-76. Therefore, the addition of the M4 gene in MHV-76 does not result in a significant difference in its ability to replicate *in vitro*.

4.1.10 Mouse Infection Studies with M4KI Virus

BALB/c mice were infected intranasally with 4x10^5 pfu of either MHV-76, MHV-68 or M4KI virus. Four mice were infected for each timepoint with one of the three viruses. Lung titrations, as described in section 2.4.18, were performed on lungs taken from mice on days 2, 6, 10, and 14 and graphed in figure 4.1.10a. As reported previously, MHV-76 is cleared more rapidly than MHV-68 from the lung (Macrae *et al.*, 2001). The lung titration graph A reveals that the M4KI virus infects the lung with the same pathogenicity as MHV-76. On day 2 the M4KI virus appears to titrate to higher levels than MHV-76. The lung titration experiment was repeated with lungs on early timepoint days 1, 2, 3, 4, and 6 as shown in figure 4.1.10a, graph B. Due to limited availability of mice, in this instance only three mice were infected for each timepoint with one of the three viruses. In both graphs A and B, M4KI titre was significantly higher than MHV-68 ($P<0.05$, Student's t-Test) on day 2 indicated by *. On graph B but not on graph A, MHV-76 titre was significantly higher than MHV-68 ($P<0.05$, Student's t-Test) on day

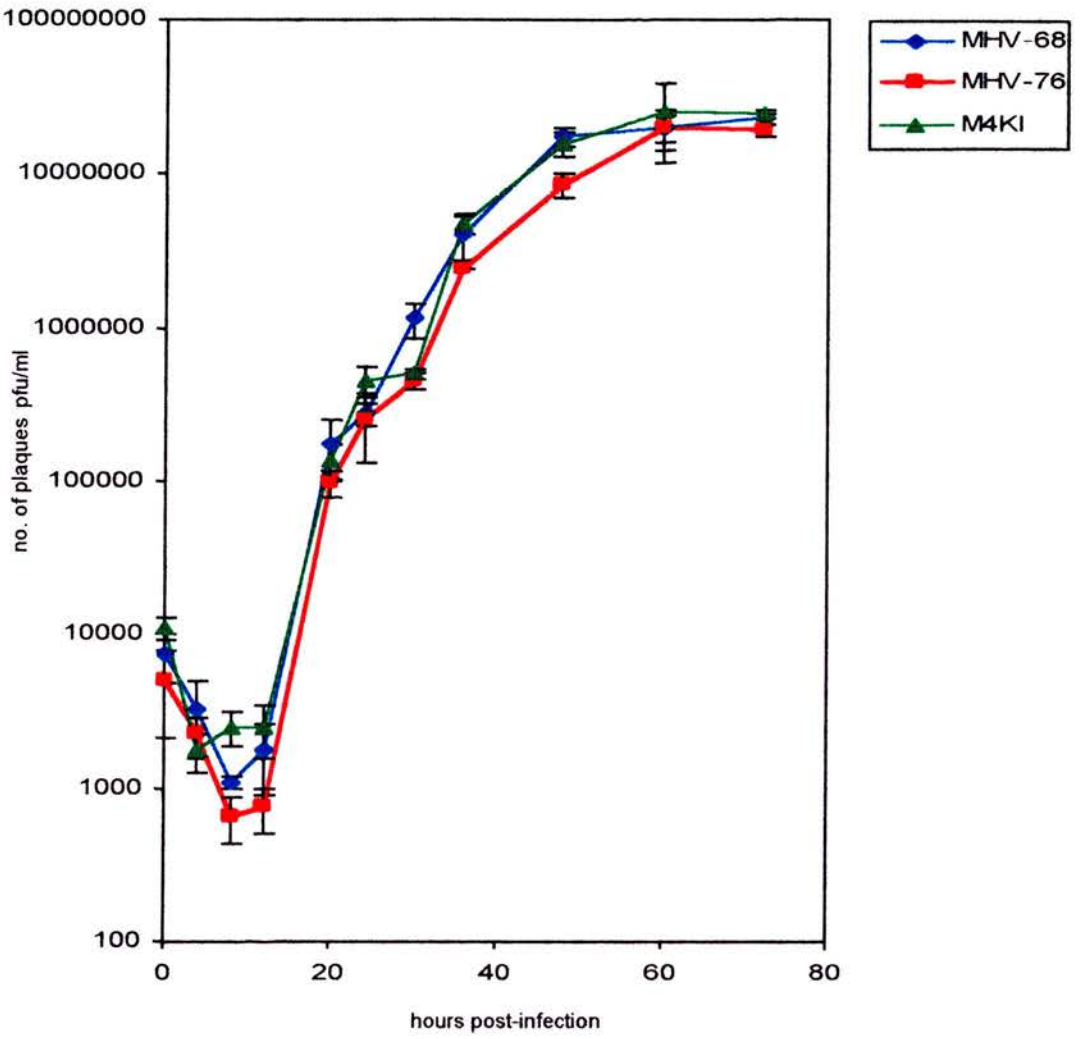
2 indicated by ~. No significant difference was observed across the other time points used.

Infective centre assays, as described in section 2.4.19, were performed on spleens taken from mice on days 6, 10, 14, 18, 21, and 31 (see figure 4.1.10b and table 4.1.10). This assay revealed, during all timepoints, that M4KI infective centres were undetectable in the spleen and there were no signs of reactivating virus in comparison to MHV-76 and MHV-68. To investigate which site within the mouse the level of M4KI virus became reduced, inhibited or undetectable, infective centre assays were performed on pooled mediastinal lymph nodes (MLN) of four mice on days 3, 6, 8, and 13. Infective virus assays were performed (as described in section 2.4.18) on the pooled MLNs to ensure that any infective centres detected were not a result of lytic virus plaques. Plaques which formed as a result of the infective virus assay were deducted from the number of infective centres (see figure 4.1.10c and table 4.1.10). Infective centres were demonstrated in the MLNs for the M4KI virus on day 3 and were severely reduced to just above detection levels on days 6 and 8. By day 10, no infective centres were detectable in the MLNs for the M4KI virus. In stark contrast, MHV-68 infective centres were not detectable on day 3 but rose to a high level from day 6 onwards. MHV-76 infective centres were detected at a high level during days 3 and 8, moderate levels on day 6 and were severely reduced on day 13. The assays showed that M4KI virus infective centres are much reduced in the MLNs in comparison to MHV-76 and MHV-68 and are not detectable in the spleen.

Splenomegaly has previously been observed in MHV-68 but not in MHV-76 spleens (Macrae *et al.*, 2001). It was interesting to examine any changes which may have occurred in the spleen with M4KI infection in comparison to MHV-68 and MHV-76. Prior to the infective centre assays, all spleens were weighed and the number of cells were counted. The spleen weights and cell counts are shown in figure 4.1.10d, in graph A and B, respectively. The weights and cell counts of M4KI spleens were not significantly different ($P<0.05$, Student's t-Test) from MHV-76. The MHV-68 spleen

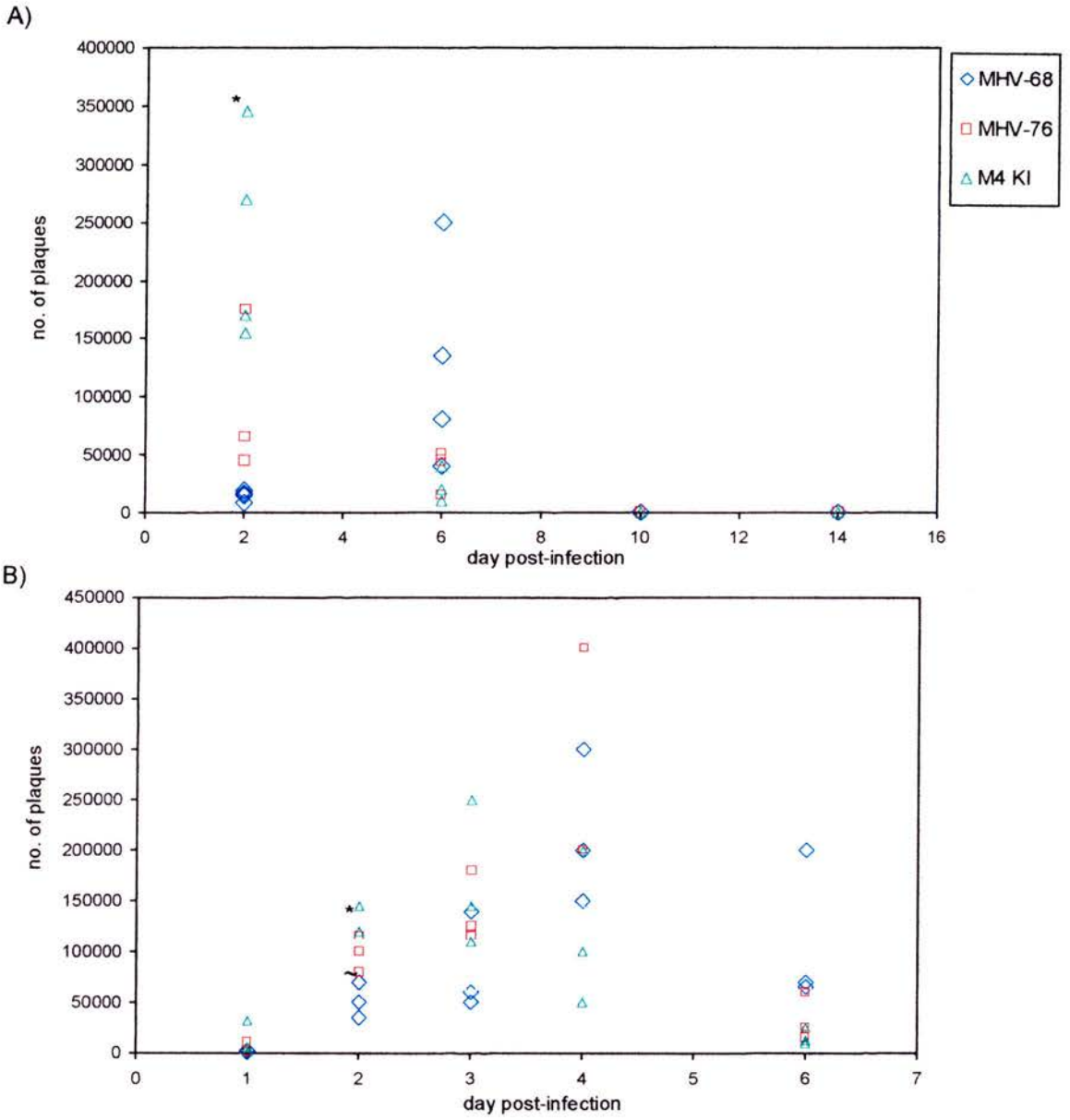
weights on days 14, 18, 21, and 31 are significantly higher than MHV-76 and M4KI ($P<0.05$, Student's t-Test) indicated by * in graph A. This is corroborated by the finding that MHV-68 spleen cell counts on days 18 and 21 are significantly higher than MHV-76 and M4KI ($P<0.05$, Student's t-Test) indicated by ~ in graph B. From this data, as would be expected, splenomegaly was not observed in the M4KI spleens. In addition, the pooled MLNs were examined for differences in cell counts, the average number of cells per MLN is shown in figure 4.1.10e. MHV-68 MLN cell counts appeared higher in days 3, 6, and 8, however, they were much lower on day 13.

Figure 4.1.9 One Step Growth Curve of M4KI Virus *In Vitro*.



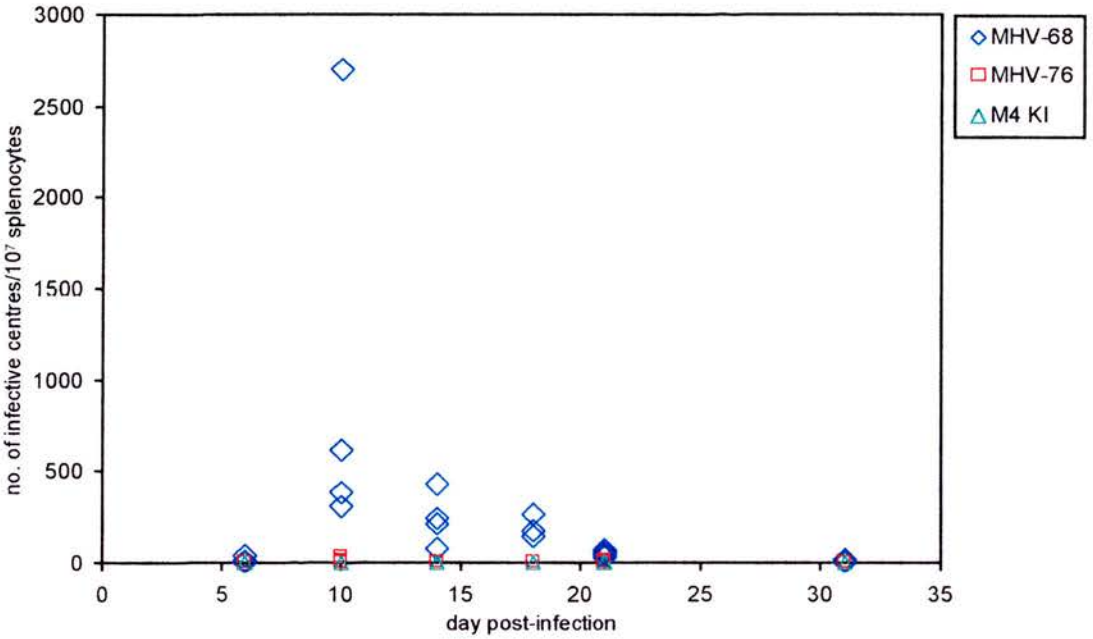
The growth kinetics of MHV-68, MHV-76 and M4KI virus were titrated on BHK-21 cells. The standard errors are shown by the black bars.

Figure 4.1.10a Lung titration of MHV-68, MHV-76 and M4KI virus.



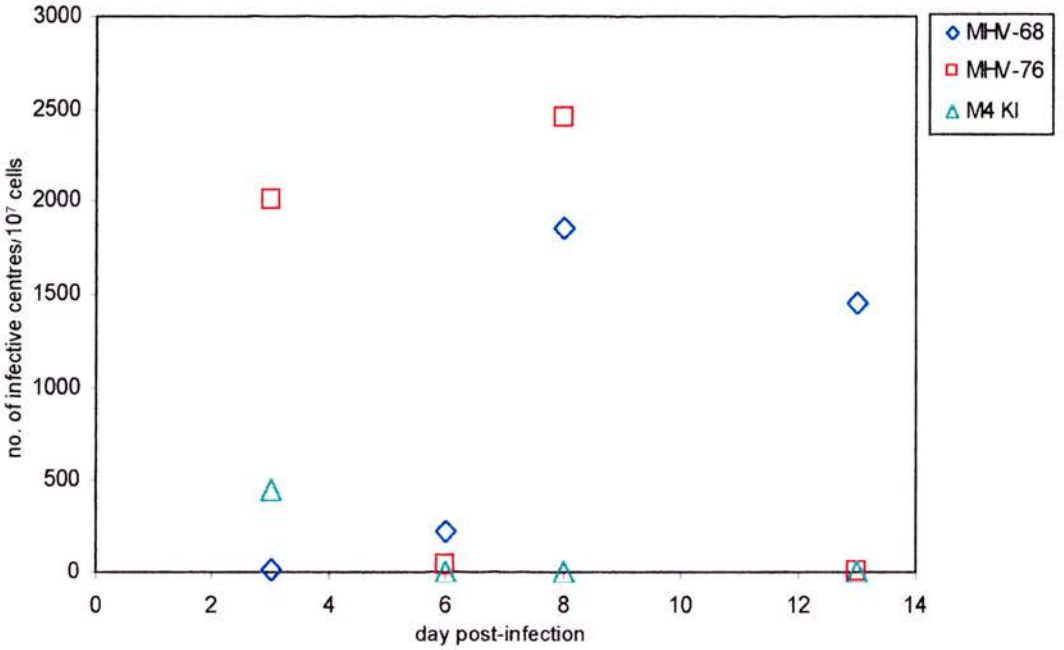
Lungs were taken from mice infected with either MHV-68, MHV-76, or M4 KI virus on days 2, 6, 10, and 14 post-infection and titrated on BHK-21 cells (see graph A). The experiment was repeated (see graph B) with lungs taken from mice on days 1, 2, 3, 4, and 6 post-infection. In both graph A and B, M4 KI titre was significantly higher than MHV-68 ($P<0.05$, Student's t-Test) on day 2 indicated by *. On graph B but not on graph A, MHV-76 titre was significantly higher than MHV-68 ($P<0.05$, Student's t-Test) on day 2 indicated by ~. No significant difference was observed across the other time points used.

Figure 4.1.10b. Spleen infective centre assay of MHV-68, MHV-76 and M4KI virus.



Infective centre assays (described in section 2.4.19) were performed on spleens of mice infected with either MHV-68, MHV-76, or M4KI virus on days 6, 10, 14, 18, 21 and 31 post-infection. The number of infective centres/10^7 splenocytes are presented in the graph and table 4.1.10. All titres for the three viruses were significantly different from day 10 onwards ($P<0.05$, Student's t-Test).

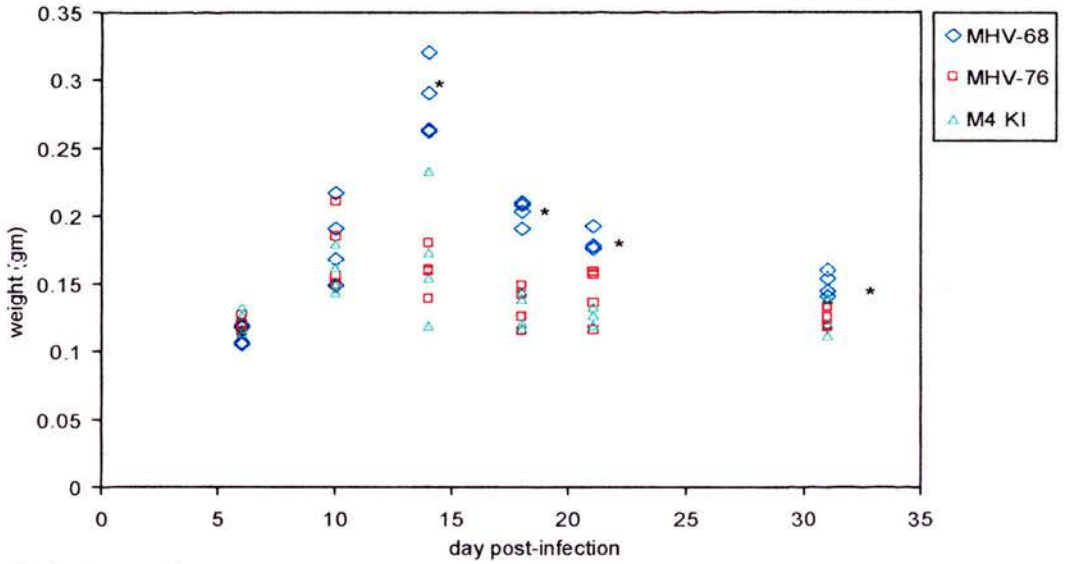
Figure 4.1.10c Mediastinal lymph node infective centre assay of MHV-68, MHV-76 and M4KI virus.



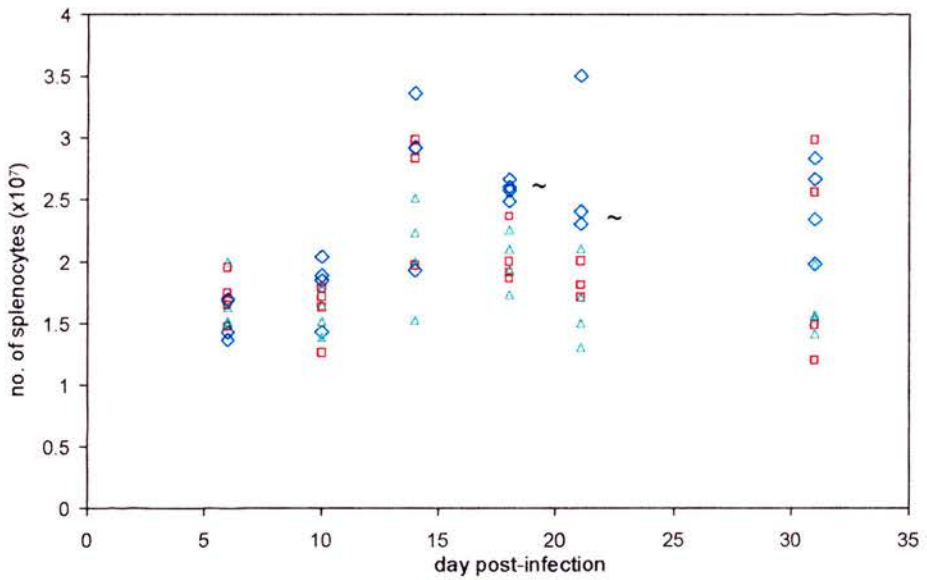
Infective centre assays (described in section 2.4.19) were performed on pooled mediastinal lymph nodes (MLN) of mice infected with either MHV-68, MHV-76, or M4KI virus on days on days 3, 6, 8, and 13. Infective virus assays (described in section 2.4.20) were performed on the MLNs, data were deducted from infective centre data and presented in the graph and table 4.1.10. The data represents the pooled MLNs of four mice.

Figure 4.1.10d Spleen measurements.

A) Spleen weights.

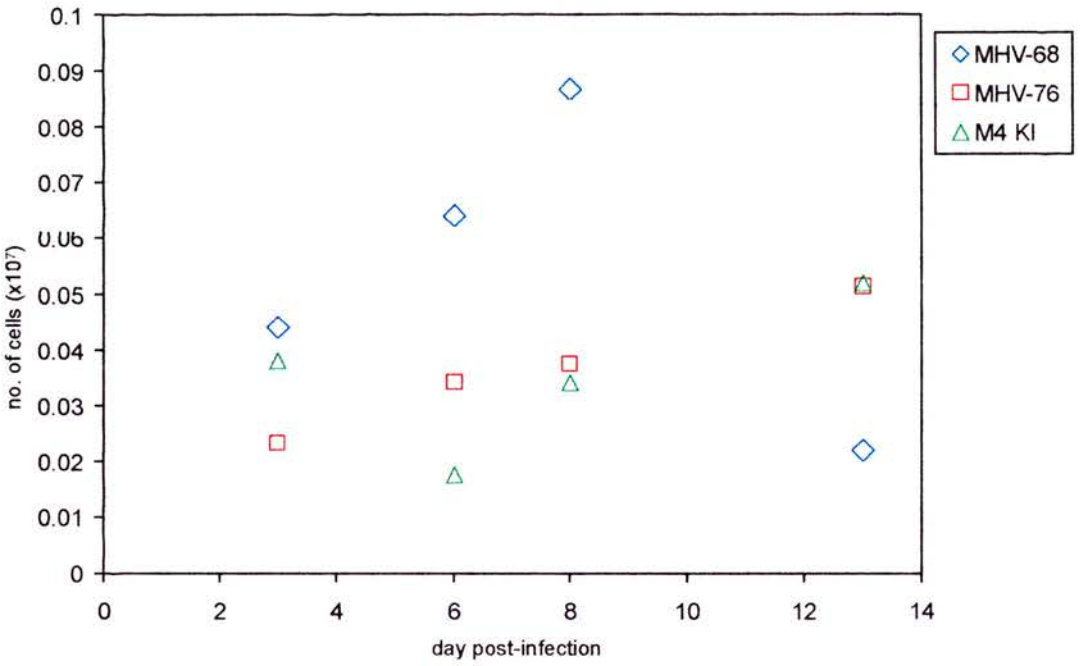


B) Spleen cell counts.



Spleens were taken from mice infected with either MHV-68, MHV-76, or M4KI virus on days 6, 10, 14, 18, 21, and 31 post-infection. The weights and cell counts of spleens were measured. Weights and cell counts of M4KI spleens were not significantly different ($P<0.05$, Student's t-Test) from MHV-76. MHV-68 spleen weights on days 14, 18, 21, and 31 are significantly higher than MHV-76 and M4KI ($P<0.05$, Student's t-Test) indicated by * in graph A. MHV-68 spleen cell counts on days 18 and 21 are significantly higher than MHV-76 and M4KI ($P<0.05$, Student's t-Test) indicated by ~ on graph B.

Figure 4.1.10e Mediastinal lymph node pooled cell counts.



Mediastinal lymph nodes (MLNs) were taken from mice on days 3, 6, 8 and 13 pooled and counted as shown in graph. The data represents the pooled MLNs of four mice and is the average number of cells/MLN.

Table 4.1.10 Spleen and MLN Infective Centre Assay Data

Day pi	Mouse number	Number of IC/10^7 Splenocytes		
		MHV–68	MHV-76	M4KI
6	1	2	1	0
	2	3	1	0
	3	7.5	1.5	0
	4	37	1	0
10	1	305	4	0
	2	380	6.5	0
	3	615	3.5	0
	4	2700	29.5	0
14	1	75	2.5	0
	2	425	2	0
	3	240	1.5	0
	4	210	1	0
18	1	265	1.5	0
	2	140	1	0
	3	170	1	0
	4	175	1	0
21	1	66	3.5	0
	2	33	8.5	0
	3	45	1	0
	4	50.5	1	0
31	1	13.5	1	0
	2	2	1	0
	3	16	1	0
	4	14.5	1	0

Day pi	No. of IC - IV/10^7 MLN Cells		
	MHV–68	MHV-76	M4KI
3	0	1590	330
6	120	40	5
8	1825	440	5
13	1430	5	0

pi – postinfection, IC – infective centres, IV – infective virus

4.1.11 FACS Analysis of M4KI Infected Cells

In order to examine the effect of the M4KI virus on B cell activation in the cells of the MLN and spleen, Fluorescent-Associated Cell Sorting (FACS) analysis was used. The cells were extracted from spleens and MLNs of mice infected with either MHV-68, MHV-76, or M4KI. 5 x 10^5 cells were used for each FACS sample and antibodies were incubated and washes were performed as described in section 2.5.2. CD19 directly conjugated to PE was used to detect the total number of B cells present and CD69 directly conjugated to FITC was used to observe differences in B cell activation. CD69 is an activation marker on B cells and T cells. The double stain was used to quantitate the number of activated B cells. FACS analysis was performed on cells from mice infected on days 6, 10, 14, 18, 21, and 31 post-infection. Data (not shown) did not indicate any overall differences in naïve and activated B cells between MHV-68, MHV-76 and M4KI during all timepoints.

4.1.12 Lung and Spleen Histopathology

The histopathology of MHV-76 infected lungs and spleens has revealed there is a more rapid development of lung inflammation compared to MHV-68 (Macrae *et al.*, 2001). As a result, the histological examination of the M4KI virus infected lungs and spleens was of interest during the *in vivo* studies. Lungs were taken from MHV-68, MHV-76, and M4KI mice on days 4, 6, 8 and 10 post-infection. Spleens were taken from MHV-68, MHV-76, and M4KI mice on days 4, 6, 8, 10, 14 and 21 post-infection. Lungs were filled with and spleens were placed in formaldehyde solution as described in section 2.4.21. Slide sections were made of these organs and they were analysed under a light microscope (kindly performed by Dr. David Brownstein). As previously observed, the lungs of MHV-76 had a more elevated inflammation during days 4 and 6 in comparison to MHV-68 (Macrae *et al.*, 2001). These characteristics were also observed in the M4KI infected lungs as shown in figure 4.1.12a. Perivascular infiltrate inflammatory cells were detected around the blood vessels on day 4 of MHV-76 and M4KI lungs. On day 6, the inflammatory cells had increased from round the blood vessel and the infiltration can be seen to spread to the lung airways. By day 8 and 10, the infection had reduced

and cleared. Germinal centre formation was detected in all infected spleens on day 8 and 10. As previously observed, the number and size of germinal centres in MHV-68 infected spleens were larger and peaked by day 14. However, this was not exhibited by MHV-76 and M4KI infected spleens as shown in figure 4.1.12b. No differences were observed between MHV-76 and M4KI infected lungs and spleens during all timepoints.

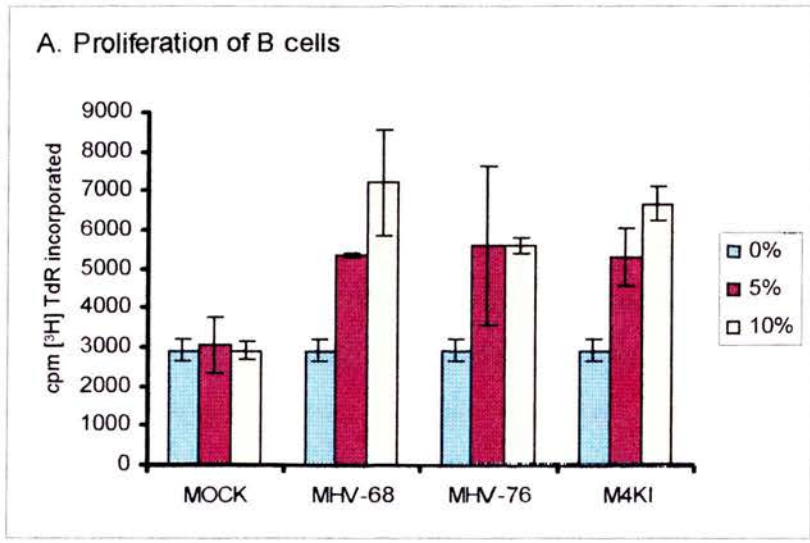
4.1.13 PCR and Southern Analysis of M4KI Infected Organs

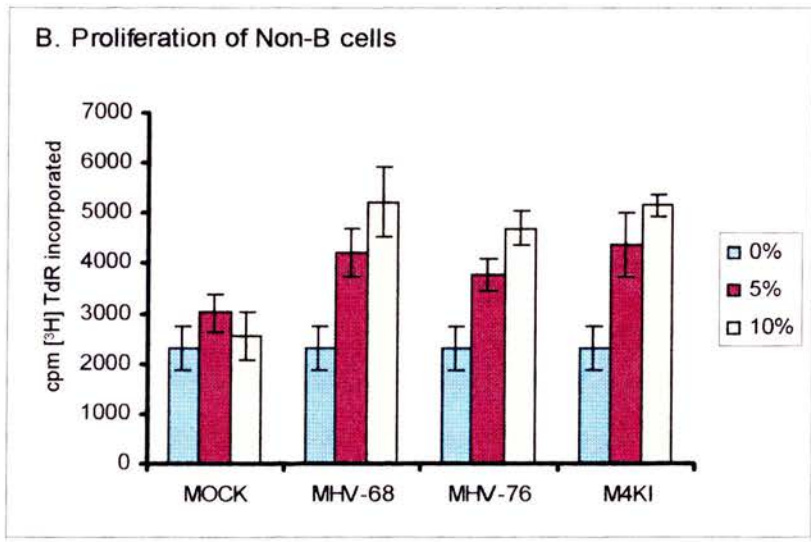
In view of the fact that no latent virus could be detected by infective centre assay in the spleens of M4KI virus infected mice, it would be of interest to examine whether lungs,spleens and mediastinal lymph nodes (MLNs) harboured the virus in long term infected mice. Furthermore, it would be particularly important to show if the M4KI virus had trafficked from the MLNs to the spleen. A Qiagen DNeasy kit was used to extract DNA from the lungs, MLNs, and spleens of three month old mice infected with either MHV-68, MHV-76 or M4KI virus. Duplicate mice were used for each virus. PCR was performed on 1μg of extracted lung, MLN, spleen, and kidney DNA with the M4A and M4B primers to detect the M4 gene. 15μl of each 100μl PCR sample were electrophoresed on a 0.7% TAE gel and Southern blotted onto a nylon membrane and fixed by UV cross-linking as described in section 2.1.21 and 2.1.22. The membrane was hybridised with a ^{32}P-labelled M4 DNA probe which did not indicate the presence of the M4 gene in any of the extracted DNA samples. The M4 probe bound to the positive control, a PCR of MHV-68 genomic DNA, but did not bind any of the DNA extracted from MHV-76 and MHV-68 organs. It has previously been demonstrated by PCR analysis that MHV-76 and MHV-68 persist long term in the spleen and lung of infected mice (Stewart *et al.*, 1998, Macrae *et al.*, 2001). The lack of detection of the M4 gene in MHV-76 and MHV-68 infected lungs and spleens would suggest there was a sensitivity problem.

4.1.14 Cell Proliferation Studies

The M4 protein is is an secreted protein which may have an immunomodulatory function. Given the nature of MHV-68 infection it is plausible that this protein may exert a growth effect on splenic cells. BHK-21 cells were infected for 24 hours with 5 pfu of either MHV-68, MHV-76, M4KI, and mock virus. Supernatants from the cells were collected, centrifuged at high speed to remove debris, and kept frozen. B cells and non-B cells were extracted from the spleens of wild type BALB/c mice and used with the supernatants for an alamar blue assay and a [^{3}H] thymidine assay as described in section 2.4.10 and 2.4.11. However, all the supernatants, including the control mock, appeared to inhibit the growth of the cells. It is possible that this was a result of the supplements used in the growth medium for the BHK-21 cells. Therefore, an alternative approach was to use another cell line, C127 cells, that did not require supplements to the growth medium. C127 cells were infected as before and supernatants were used for the same assay. Different supernatant concentrations were used at 0%, 5%, and 10% of the total culture volume. The amount of incorporated tritiated thymidine by the cells was measured. MHV-68, MHV-76, and M4KI supernatants stimulated the proliferation of B cells and non-B cells in comparison to the mock supernatant (see figure 4.1.14). However, no differences were observed in the level of proliferation between the three viruses.

Figure 4.1.14 Cell Proliferation Assay.





Proliferation studies were performed on A) B cells and B) non-B cells (described in section 2.4.11). Three concentrations (0%, 5%, and 10%) of virus-infected cell supernatant were used. The amount of tritiated thymidine ([3H] TdR) incorporated by the cells was measured by a scintillation counter.

4.2 Recombinant Virus Discussion

The generation of a mutant virus is a desirable way to explore the function of the gene of interest in its natural host environment. The advent of recombinant DNA technology and PCR has allowed the generation of mutants to be a more specific process. However, before recombinant DNA technology existed, mutant herpesviruses were isolated by phenotyping and this was initiated by herpes simplex virus (HSV). Classical approaches in creating mutant HSV include altered plaque phenotype (Ejercito *et al.*, 1968), drug resistance (Kitt *et al*, 1964), host-range properties (Aurelian, 1964), and temperature-sensitive mutants (Brown *et al.*, 1973). Targeted disruption of alphaherpesvirus and betaherpesvirus genes has been shown to be extremely beneficial in elucidating the functions of the genes (Balan *et al.*, 1994, Vieira *et al.*, 1998). There has been some success in the generation of gammaherpesviruses with disrupted genes, however, more difficulties have been encountered with the manipulation process (Borza & Hutt-Fletcher, 1998, Wang & Hutt-Fletcher, 1998). Recognition of homologous recombinants is facilitated by the use of a visual marker. For our studies, we took advantage of the GFP cassette, a green fluorescence emitting protein under the control of a constitutive promoter (Zolotukhin *et al.*, 1996). This marker has been successfully used in a number of recombinant studies (Vieira *et al.*, 1998, Prichard 1999, Duboise 1996). An alternative visual marker which has been successfully used is the β-galactosidase gene (Simas *et al.*, 1998, van Dyk *et al.*, 2000, Clambey *et al.*, 2000). Selectable markers that we could have made use of include the neomycin resistance gene (Grassman & Fleckenstein, 1989), the HSV-1 thymidine kinase gene (Post *et al.*, 1981), the hygromycin phosphotransferase gene (Marchini *et al.*, 1992), and the *E.coli* guanosine phosphoribosyl transferase (*gpt*) gene (Greaves *et al.*, 1995),

As mentioned previously, murine models of both human alphaherpesvirus and betaherpesvirus infection exist which are invaluable for recombinant virus studies. There are many advantages in using mouse models, including the vast amount of genetic information already known about them and their cost efficiency. MHV-68 is a gammaherpesvirus which infects mice and can grow permissively in tissue culture,

unlike EBV and KSHV, and can be utilised for the generation of recombinant viruses for *in vivo* studies. The MHV-68 model has recently be used to generate recombinant viruses, including viruses lacking the M1 and cyclin D genes (Simas *et al.*, 1998, Clambey *et al.*, 2000, Hoge *et al.*, 2000, van Dyk *et al.*, 2000). It is clear that an important series of experiments will involve "knocking out" the MHV-68 genes of interest one by one and analysing their pathogenesis in the virus's natural host.

4.2.1 Knocking M4 Out of MHV-68

A GFP recombination construct encoding homologous flanking regions to the M4 gene was used to target the disruption of the MHV-68 M4 gene. The excised GFP construct with M4 flanking regions was co-transfected with MHV-68 DNA into cells and produced green fluorescent plaques, indicative of the recombinant virus. A number of wild type (non-green) plaques were visible, therefore, rounds of plaque purification were performed until only recombinant plaques were observed. PCR analysis was performed on recombinant viral DNA extracted from cells that had been infected with a single isolated green plaque. This revealed that the M4 gene was present and the recombination event had not disrupted the M4 gene. If time had allowed, Southern analysis could have been performed with the recombinant viral DNA to detect the exact location of the non-homologous recombination. However, for my particular project objective, it would have been time consuming and may not necessarily have revealed any useful information.

A recombinational "hot-spot" has been proposed at the left hand end of the MHV-68 genome (Adler *et al.*, 2000, Roy, unpublished observation, Wakeling, 2001). It is possible that the non-homologous recombination which occurred in this instance may have been in this "hot spot". Furthermore, like HVS, this region of the MHV-68 genome is a potential site for the virus origin of replication resulting in a more accessible part of the genome (Bowden *et al.*, 1997, Grassman & Fleckenstein, 1989).

Another possibility for the failure of the homologous recombination is that the 600bp flanking regions were not long enough to allow such an event to take place. With hindsight, increasing the flanking regions to 2-3kb may have resulted in a successful homologous recombination. However, this may not have altered matters as it has been demonstrated that even with 2kb flanking regions homologous recombination does not necessarily occur (Wakeling, 2001).

Interestingly, homologous and non-homologous recombination may have occurred simultaneously but the non-homologous recombination event conferred a growth advantage to that particular virus. Therefore, during the time spent purifying the green virus, the non-homologous recombinant virus may have competed out the homologous recombinant virus.

4.2.2 Knocking M4 Into MHV-76

Despite the problems encountered, it would still be invaluable to generate a recombinant virus for *in vivo* studies. The alternative strategy used was the addition of the M4 gene into MHV-76, a deletion mutant of MHV-68 that lacked a number of left hand genes including M4 (Macrae *et al.*, 2001). The M4 "knock-in" recombination construct was used to insert the M4 gene into the left hand end of the MHV-76 genome via homologous recombination. Once again green fluorescent plaques were observed indicating recombination had been successful. The green plaques were purified and upon lack of wild type detection, PCR analysis was performed immediately on the recombinant viral DNA to ensure a homologous recombinant virus was not competed by a non-homologous recombinant alternative. PCR analysis indicated homologous recombination had been successful and a Southern analysis was performed on the M4KI virus to ensure purity of the virus. Southern analysis revealed that all of the bands which distinguished MHV-68, MHV-76, and M4KI virus were present. As previously observed, the laddering effect of the DNA is indicative that the specific DNA being probed for is adjacent to the terminal repeats which consists of repetitive DNA

(Efstathiou *et al.*, 1990b). It was apparent from the bands on the Southern blot that laddering may alter the sizes and visibility of some of the bands.

Before pursuing *in vivo* studies, it was necessary to ensure the M4KI virus was transcribing the M4 gene. This was confirmed by two methods. RT-PCR was performed on cDNA prepared from RNA extracted from M4KI infected cells. In addition, northern analysis was performed on the M4KI RNA. Both these methods detected M4 transcript expression. Moreover, the transcript was indistinguishable in size from MHV-68 indicating wild type transcription.

The growth kinetics of the M4KI virus *in vitro* were analysed and compared to MHV-68 and MHV-76 using a single step growth curve over 72 hours post-infection. MHV-68 replicated efficiently and attained maximal titres at around 60 hours post-infection and the growth kinetics of MHV-76 were similar to MHV-68 (Sunil-Chandra, 1991, Macrae *et al.*, 2001). The addition of the M4 gene in MHV-76 did not alter the virus's ability to replicate *in vitro*. This does not implicate a major role for M4 during *in vitro* replication.

One of the major aims of the project was to perform *in vivo* studies with a M4 recombinant virus. During MHV-68 infection of the lungs, virus titres peak at around day 7 and are cleared by day 10 by the immune system (Ehtisham *et al.*, 1993). In a similar experiment with MHV-76, the virus is cleared more rapidly from the lung in comparison to MHV-68 (Macrae *et al.*, 2001). It was of interest to observe the effects of the M4KI virus on the productive replication in lungs. Mice were infected with either MHV-76, MHV-68 or M4KI virus and the virus titres in the lungs were analysed at various times post-infection by plaque assay. The M4KI virus replicated productively *in vivo* with the same overall kinetics as MHV-76. Therefore, the presence of the M4 gene did not reduce or increase the clearance of the virus from the lungs in comparison to MHV-76 and, furthermore, did not restore the kinetics and productive infection to that

observed with MHV-68. This indicates that the difference between MHV-76 and MHV-68 is not due to lack of M4.

Splenomegaly is characteristic of MHV-68 infection but, interestingly, during MHV-76 infection this is dramatically reduced and a lower number of infective centres are observed (Suni-Chandra *et al.*, 1992, Macrae *et al.*, 2001). This suggests a role for the genes at the left hand end of the MHV-68 genome in splenomegaly and B cell latency. It is not clear which of the genes are involved, therefore, the effects of the M4KI virus on splenomegaly were observed and compared to MHV-76 and MHV-68. The spleens of mice infected with either MHV-76, MHV-68 or M4KI virus were assayed for the presence of virus at various times post-infection. The characteristic splenomegaly and infective centres were observed with MHV-68 infection and a significant reduction was observed with MHV-76 infection. The M4KI-infected spleen weights and cell counts were similar to MHV-76. Interestingly, splenomegaly was not observed with M4KI-infected spleens and no infective centres were apparent at any time post-infection. These observations raised two important issues. Firstly, at which point within the mouse does the level of M4KI virus became reduced, inhibited or undetectable. Secondly, is the virus present in the spleen but at such low numbers that the sensitivity of the infective centre assay cannot detect it. The MLNs are involved with the trafficking of virus to the spleen. Therefore to investigate these issues, infective centre assays were performed on the mediastinal lymph nodes in addition to the spleen. In contrast to MHV-68 and MHV-76, M4KI virus infective centres were much reduced in the MLNs and were not detectable by day 10. It is apparent that the addition of the M4 gene into MHV-76 results in a dramatic reduction in the latent infection of MLNs and infection is undetectable in the spleen by infective centre assay. Thus, the M4 gene product has a role in inhibiting the movement of the virus from the MLNs to the spleen and may interfere in the establishment of latency. It is possible that the M4 gene is involved in and may inhibit reactivation from latency. However, M4 has been shown to be a lytic transcript in S11 cells, thus the protein is not likely to be latency-associated (Husain *et al.*, 1999).

MHV-76 and MHV-68 maintain long-term persistence in the spleen and lung and this has been shown by PCR analysis (Sunil-Chandra *et al.*, 1992, Stewart *et al.*, 1998, Macrae *et al.*, 2001). The M4KI infective centre studies suggest that the virus may not infect or establish latency in the spleen. However, if the M4KI virus is present in the spleen it remains to be determined whether the virus is cleared or persists. PCR analysis was performed on organs of M4KI infected mice at 3 months post-infection and was unable to detect presence of the viral DNA in any of the organs. However, MHV-68 and MHV-76 infected organs were used as positive controls and the PCR could not detect the presence of viral genome either which suggests there is a sensitivity problem. Either the DNA extraction process is not efficient or very low amounts of viral DNA are present. The PCR analysis is sensitive to 1-10 copies of the viral genome in 1μg of high molecular weight DNA.

A histopathological study shows that greater inflammatory response occurs in the lungs of MHV-76 infected mice than in MHV-68 infection (Macrae *et al.*, 2001). Since the M4KI virus appears to have significant differences in splenomegaly and latent infection of B cells when compared to MHV-76, a histopathological study was performed. The study revealed there was no difference in the inflammatory response in the lungs of MHV-76 and M4KI virus. No differences were observed in the histopathology of MHV-76 and M4KI spleens.

The M4 gene is predicted to be a putative viral-encoded cytokine and thus may have a immunomodulatory role involving B cell proliferation. The presence of the M4 gene in MHV-76 may influence the role of B cells in trafficking of the virus. Thus, B cell activation of MLN and spleen cells was analysed by FACS analysis and this revealed there were no significant differences in B cell activation between the three viruses. Furthermore, M4KI virus supernatant did not appear to stimulate the growth of splenic B cells and non-B cells *in vitro* compared to that of MHV-76.

With hindsight, during the generation of the M4KI virus, a control M4KI virus should have been generated which would have only contained the GFP selectable marker. This would ensure that the M4KI virus phenotype alteration is not a result of the neighbourhood effect of the GFP gene and instead due to the M4 gene.

Rescue viruses are vitally important in determining whether the phenotypic alterations of a recombinant virus is due to the specific recombination event or a random mutation elsewhere in the genome. In the MHV-68 M1 and cyclin D "knock-out" virus studies, restoration of the removed DNA sequence demonstrated that no additional mutations were present in the MHV-68 genome (Clambey *et al.*, 2000, Hoge *et al.*, 2000). In the MHV-76 studies, the deleted sequences at the left hand end of the genome was restored to determine whether the differences observed between MHV-76 and MHV-68 were due to this missing region (Macrae *et al.*, 2001).

If time had permitted, the generation of a M4 rescue virus would be invaluable to determine if the phenotypic effects of the M4KI virus were due to the presence of the M4 gene. The M4 rescue virus would remove the M4 gene at the left hand end of the M4KI virus and be compared to wild type MHV-76 *in vivo* and *in vitro*.

4.2.3 Recombinant Virus Strategies

Although the generation of a M4 "knock-in" virus was successful and beneficial for our studies, it would have been desirable to achieve the initial goal of a M4 "knock-out" virus. Ultimately, a system that distinguished homologous from non-homologous recombination would be favourable. The purification of recombinant green plaques from wild-type plaques was time consuming and unsuccessful, thus bypassing this procedure would be of great assistance.

Cosmid libraries have been used as an alternative approach in generating recombinant viruses (Cunningham & Davison, 1993; de Wind *et al.*, 1990; van Zijl *et al.*, 1988). Advantages of this system are that entire virus genomes are cloned into overlapping

cosmid clones and genes of interest are directly manipulated within bacteria. Overlapping cosmids are linearised and the intact mutant virus genome can be reassembled by homologous recombination of the individual genomic fragments in permissive cells. This system does not require insertion of a selectable marker thus eliminating the possibility of neighbourhood effects on the DNA surrounding the gene of interest. A disadvantage to this system is that repetitive sequences such as terminal and internal repeats tend to be unstable in bacteria (Gray & Kaerner, 1984; Quinn & McGeoch, 1985; Weller *et al.*, 1985).

Recently, there has been a heightened interest in the use of bacterial artificial chromosome (BAC) technology in generating herpesvirus recombinants. The advantages of the BAC system are that they can harbour up to 300kb of DNA, are easy to handle, less time consuming and are relatively stable. Large virus genomes, including HSV, EBV, pseudorabies virus, MCMV, and HCMV, have been inserted into single BACs thus allowing the genetic manipulation of viral genes using bacterial recombination machinery (Messerle *et al.*, 1997, Horsburgh *et al.*, 1999, Delecluse *et al.*, 1998, Smith & Enquist, 1999, Borst *et al.*, 1999). BACs containing the viral genomes are maintained in *Escherichia coli* and viral progeny are produced via transfection of the BAC plasmid into eukaryotic cells. This system has been used to produce a MHV-68 BAC which will facilitate the generation of MHV-68 recombinants (Adler *et al.*, 2000). An ORF 4 recombinant virus and its revertant virus have been generated using this BAC system (Adler *et al.*, 2000). Similar to the cosmid system, repetitive sequences are unstable in the BAC system and this was shown by spontaneous recombination events which occurred within the internal and terminal repeats of the MHV-68 genome (Adler *et al.*, 2000).

Chapter Five:

Conclusion

5.1 Conclusion and Potential Function of M4

Many viral immunomodulatory proteins exist to subvert the host's immune response (Spriggs, 1996, Alcami & Koszinowski, 2000, Lalani *et al.*, 2000, McFadden & Murphy, 2000, Tortorella *et al.*, 2000). A number of these proteins are encoded by viral genes with sequence homology to cellular genes. Their existence suggests that at some point viruses abducted and modified genes from the host that would be of benefit for themselves. However, there are many viral genes without sequence similarity to cellular genes. Viral genes with no cellular homologues may be examples of genes with as yet unidentified homologs in the host.

The MHV-68 M4 gene is a novel gene with an unknown function (Virgin *et al.*, 1997). It is likely that MHV-68 harbours the M4 gene to assist its own survival and may function to evade the host immune response. The M4 gene is predicted to encode a secreted cytokine with an immunomodulatory role and is an example of a gene that may have an unidentified cellular homologue.

The initial characterisation of the M4 gene of MHV-68 is demonstrated in this thesis. Sequence analysis revealed that the M4 gene has seven potential glycosylation sites and a heparin sulphate binding domain. A secretory signal exists near the N-terminus of the M4 gene. The half of the M4 protein nearest the N terminus was expressed and used as an antigen in rabbits to raise antibody production. The antibody bound to a protein of ~44kDa, the predicted size of the M4 protein. M4 transcriptional analysis *in vitro* revealed that M4 is expressed as a 1.3kb transcript during the early and late phase of MHV-68 lytic infection. The M4 gene was inserted into MHV-76, a deletion mutant of MHV-68, to generate a M4 knock-in (M4KI) virus. Growth kinetics studies *in vitro* revealed the M4KI virus growth curve is similar to MHV-68 and MHV-76. *In vivo* mouse studies revealed that the M4KI virus replicates in the lung with the same kinetics as MHV-76. M4 KI virus infective centres were much reduced in the mediastinal lymph nodes in comparison to MHV-76 and MHV-68, and were not detectable in the spleen. Thus, it appears that the M4KI virus inhibits the trafficking of the virus from the

mediastinal lymph nodes to the spleen. Generation of a rescue virus would confirm whether this anti-pathogenic effect is due to the addition of M4 or a random mutation elsewhere in the genome.

Since commencing the writing of my thesis, further studies in our laboratory with the M4KI virus have revealed additional data. Recently, it has been demonstrated that mice deficient in the type I interferon receptor (IFN-α/β $R^{-/-}$) are highly susceptible to MHV-68 infection compared to wild type mice (Dutia *et al.*, 1999). MHV-76 infection of the type I IFN R knock-out mice reveals that this virus is more pathogenic than MHV-68 and results in a lethal disease (A. Townsley, personal communications). Interestingly, the same experiment using the M4KI virus results in a non-pathogenic infection, that is, addition of the M4 gene disrupts the pathogenicity observed with MHV-76 infection (A. Townsley, personal communications). Furthermore, genes present in MHV-68 and not in MHV-76, candidates being the left hand end genes, clearly interfere with the type I interferon system. These experiments provide evidence for an anti-pathogenic role for M4.

The anti-pathogenic mechanism by which the M4 protein functions is unknown. It is possible that the M4 protein may function as a natural killer cell activator or a non-specific T cell activator. Clearly, M4 on its own is a disadvantage to the virus as viral clearance is accelerated. This indicates that M4 must interact with another virus protein absent in MHV-76. A candidate gene is the M3 gene that encodes a chemokine binding protein and may be involved in downregulating the immune response (Parry *et al.*, 2000, van Berkel *et al.*, 2000). During virus infection, the M3 and M4 genes may co-operate to evade the host's immune response. A M3 and M4 double knock-in virus would be most beneficial in elucidating the interactions between the two genes. Deleting the M4 gene from MHV-68 would still be desirable as the virus would still retain the other M genes. A M4 knock-out virus is likely to exhibit a different phenotype from the M4KI virus. The recent generation of a MHV-68 BAC will be of much assistance in achieving this aim (Adler *et al.*, 2000). During MHV-68 infection, B cell proliferation is clearly

important. FACS analysis and proliferation studies of B cells did not reveal any association between M4 and B cell growth. Also, the M4KI virus appeared to inhibit viral persistence. Hence, it is possible that M4 has a role in downregulating B cell infection.

In conclusion, M4 is a potential cytokine that may harbour an anti-pathogenic function *in vivo*. The mechanism by which M4 functions remains to be elucidated. Its role in the pathogenesis of MHV-68 *in vivo* may involve interaction with other viral encoded proteins.

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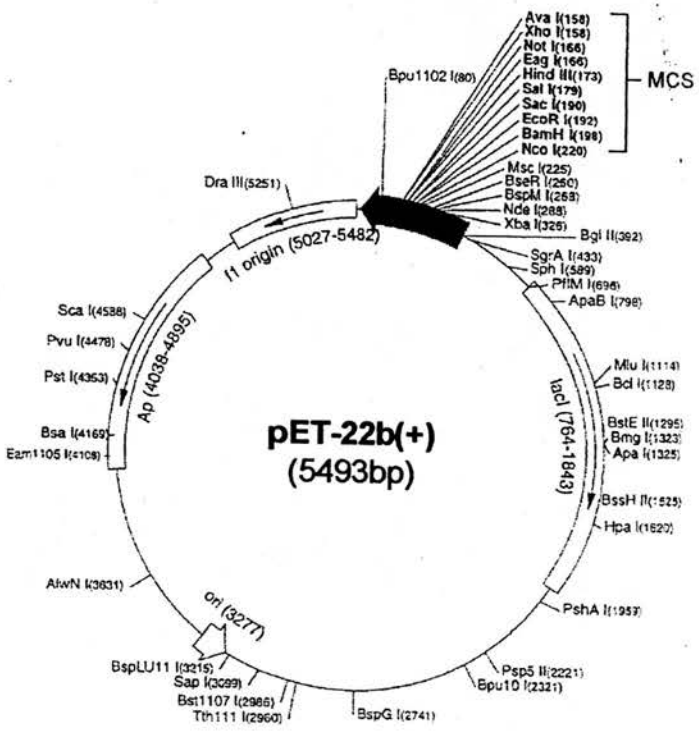
Appendix 1. Cell lines.

Cell lines	Properties
Baby Hamster Kidney cell line (BHK-21)	Immortal fibroblastoid cell line (Stoker & MacPherson, 1961).
COS (COS-7)	SV40 transformed fibroblastoid cell line (Gluzman, 1981).
293	Human fibroblastoid cell line (Graham *et al.*, 1977).
αβSV1	Murine embryo fibroblast cell line which lacks the type I interferon receptor (kindly provided by Dr. J. P. Stewart).
C127 (ATCC CRL 1616)	Mouse epithelial cell line derived from a mammary carcinoma (Lowy *et al.*, 1978).

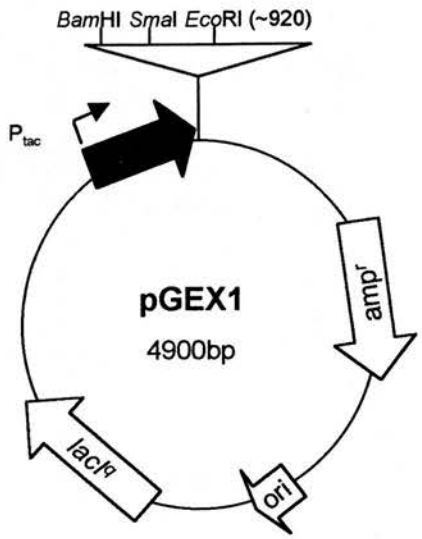
Appendix 2. pET-22b(+) vector.

pET-22b sequence landmarks	
T7 promoter	361-377
T7 transcription start	360
pelB coding sequence	224-289
Multiple cloning site	158-225
Histidine coding sequence	140-127
T7 terminantor	26-72
lacI coding sequence	764-1843
pBR322 origin	3277
B-lactamase (Ap)	4038-4895
F1 origin	5027-5482



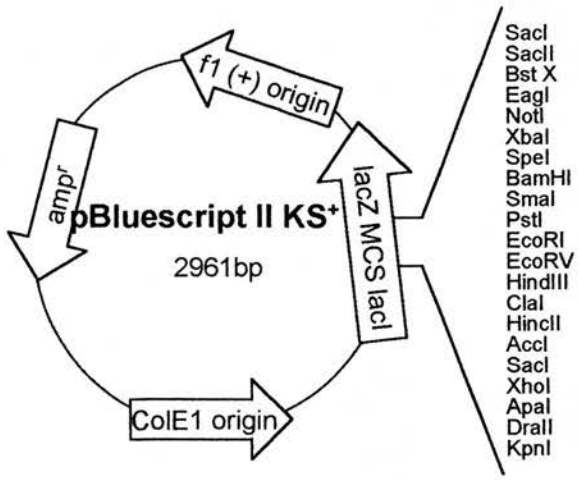
pET-22b(+) vector (Novagen)

Appendix 3. pGEX1 vector.



pGEX1 vector (Pharmacia)
P_{tac}: promoter
Amp^r: ampicillin resistance gene
Ori: origin of replication
$Lacl^q$: *lac* repressor
GST: glutathione-S-transferase

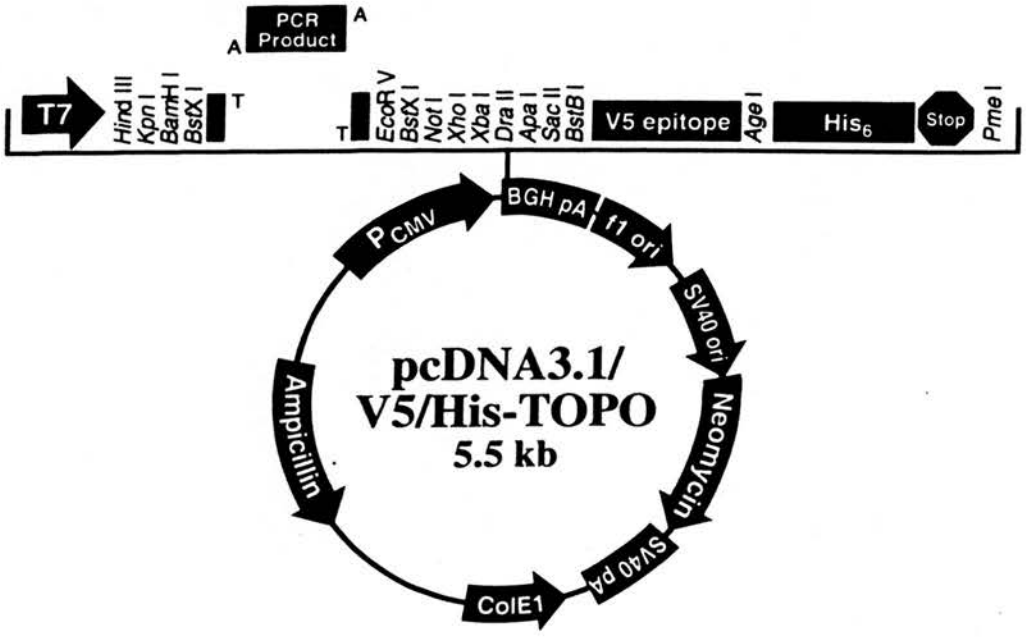
Appendix 4. pKS vector.



pBluescript II KS(+) vector (Stratagene)

f1 (+) origin: f1 origin of replication
ColE1 origin: origin of replicationLacI: Certain sequences of the LacI gene have been deleted, non-functional.
LacZ: Provides α-complementation for blue/white colour selection. An inducible lac promoter upstream from the lacZ gene permits fusion protein expression with the β-galactosidase gene product.
MCS: Multiple cloning site
amp^R: Ampicillin resistance gene

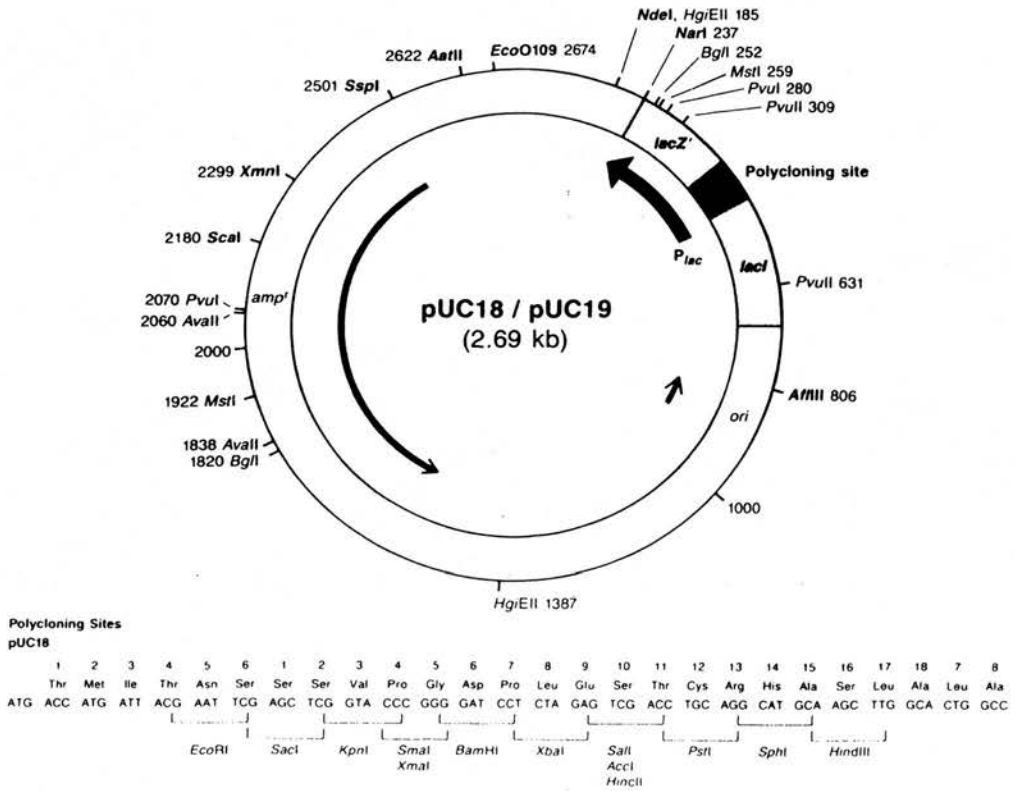
Appendix 5. pcDNA3.1/V5/His TOPO vector.



pcDNA3.1/V5/His TOPO vector (Invitrogen)

P_{CMV}: CMV immediate-early promoter
T7: T7 promoter
His_6: polyhistidine tag sequence
BGH pA: bovine growth hormone polyadenylation signal
f1 ori: f1 origin of replication
SV40 ori: SV40 early promoter and origin
neomycin: neomycin resistance gene
SV40 pA: SV40 polyadenylation signal
ColE1: origin of replication

Appendix 6. pUC18 vector.



pUC18 vector (Stratagene)

LacI: Certain sequences of the LacI gene have been deleted, non-functional.
LacZ: Provides α-complementation for blue/white colour selection. An inducible lac promoter upstream from the lacZ gene permits fusion protein expression with the β-galactosidase gene product.
amp^r: Ampicillin resistance gene
ori: origin of replication

Appendix 7. Antibodies.

ANTIBODY	CONCENTRATION	SUPPLIER
Anti-rabbit IgG biotin conjugate.	1: 80000 - Western	Sigma
Anti-mouse IgG biotin conjugate.	1:80000 - Western	Sigma
Monocolonal anti-polyHistidine clone HIS-1.	1: 500 - Western	Sigma
Anti-GST polyclonal antibody.	1:100 - Western	Stewart, unpublished
Anti-MHV-68 polyclonal antibody. Raised against MHV-68 infected rabbit kidney cells. Reacts with lytic cycle polypeptides.	1:1000 - Western 1:1000 - IF	(Sunil-Chandra *et al.*, 1992a)
FITC-labelled swine anti-rabbit.	1:50 - IF	Dako
Hamster monoclonal antibody to mouse CD69 conjugated to FITC.	1:50 - FACs	Serotec
Rat monoclonal antibody to mouse CD19 conjugated to PE.	1:20 - FACs	TCS Biologicals
Control hamster IgG conjugated to FITC.	1:85 - FACs	TCS Biologicals
Control rat IgG conjugated to PE.	1:85 - FACs	Pharmingen

IF – immunofluorescence, FACs - Flow Cytometric Analysis